Phase-Locking Requires Efficient Ca^{2+} Extrusion at the Auditory Hair Cell Ribbon Synapse

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Proper perception of sounds in the environment requires auditory signals to be encoded with extraordinary temporal precision up to tens of microseconds, but how it originates from the hearing organs in the periphery is poorly understood. In particular, sound-evoked spikes in auditory afferent fibers *in vivo* are phase-locked to sound frequencies up to 5 kHz, but it is not clear how hair cells can handle intracellular Ca^{2+} changes with such high speed and efficiency. In this study, we combined patch-clamp recording and two-photon Ca^{2+} imaging to examine Ca^{2+} dynamics in hair cell ribbon synapses in the bullfrog amphibian papilla of both sexes. We found that Ca^{2+} clearance from single synaptic ribbons followed a double exponential function, and the weight of the fast component, but not the two time constants, was significantly reduced for prolonged stimulation, and during inhibition of the plasma membrane Ca^{2+} ATPase (PMCA), the mitochondrial Ca^{2+} uptake (MCU), or the sarcolemma/endoplasmic reticulum Ca^{2+} ATPase (SERCA), but not the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Furthermore, we found that both the basal Ca^{2+} level and the Ca^{2+} rise during sinusoidal stimulation were significantly increased by inhibition of PMCA, MCU, or SERCA. Consistently, phase-locking of synaptic vesicle releases from hair cells was also significantly reduced by blocking PMCA, MCU, or SERCA, but not NCX. We conclude that, in addition to fast diffusion mediated by mobile Ca^{2+} buffer, multiple Ca^{2+} extrusion pumps are required for phase-locking at the auditory hair cell ribbon synapse.

Key words: Ca^{2+} clearance; Ca^{2+} extrusion; hair cell; phase-locking; ribbon synapse

Significance Statement

Hair cell synapses can transmit sound-driven signals precisely in the kHz range. However, previous studies of Ca^{2+} handling in auditory hair cells have often been conducted in immature hair cells, with elevated extracellular Ca^{2+} concentration, or through steady-state stimulation that may not be physiologically relevant. Here we examine Ca^{2+} clearance from hair cell synaptic ribbons in a fully mature preparation at physiological concentration of external Ca^{2+} and at physiological temperature. By stimulating hair cells with sinusoidal voltage commands that mimic pure sound tones, we recapitulated the phase-locking of hair cell exocytosis with an *in vitro* approach. This allowed us to reveal the Ca^{2+} extrusion mechanisms that are required for phase-locking at auditory hair cell ribbon synapses.

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Introduction

Sound perception requires acoustic signals to be encoded with extraordinary temporal precision up to $\sim 10 \mu\text{s}$ (Hudspeth, 1997). One hallmark embodying the auditory temporal precision is phase-locking, a phenomenon that the timing of spikes fired by single auditory neurons is synchronized and therefore phase-locked to acoustic cycles (Heil and Peterson, 2017). Phase-locking occurs in many neurons along the auditory pathways (Paolini et al., 2001), including auditory afferent fibers, for essentially all vertebrate species (Rose et al., 1967; Hillery and Narins, 1984; Rose and Weiss, 1988; Köppl, 1997; Taberner and Liberman, 2005). Despite the fact it has been studied extensively for decades, how phase-locking originates from auditory afferent fibers is still poorly understood (Avisar et al., 2007; Heil and Peterson, 2017).

In the vertebrate hearing organs, auditory afferent fibers connect to hair cells through ribbon synapses where hair cells release neurotransmitter glutamate via exocytosis, which binds and activates AMPA-type glutamate receptors in auditory afferent fibers (Glowatzki and Fuchs, 2002; Schnee et al., 2013; Coate et al., 2019). Given that phase-locking can be achieved in auditory afferent fibers for frequencies up to ~ 5 kHz (Taberner and Liberman, 2005), exocytosis from hair cells must be tightly controlled with sufficient temporal precision required. It is well established that exocytosis from hair cells is Ca^{2+} -dependent, and Ca^{2+} ions enter hair cells through voltage-gated L-type Ca^{2+} channels (Beutner et al., 2001; Roux et al., 2006; Li et al., 2009). It has been shown that the coupling distance between Ca^{2+} channels and docked synaptic vesicles is in the nanometer range (Brandt et al., 2005; Goutman and Glowatzki, 2007; Graydon et al., 2011; Cho and von Gersdorff, 2012; Pangršič et al., 2015). Nanodomain coupling between Ca^{2+} channels and docked synaptic vesicles allows for short synaptic delays that are better suited for phase-locking to acoustic signals (Li et al., 2014; Johnson et al., 2017). However, phase-locking of spikes in auditory afferent fibers also requires these Ca^{2+} ions to be cleared away rapidly and efficiently, a process that is not well understood. Furthermore, it has been shown that the Ca^{2+} rise at hair cells can be enhanced through Ca^{2+} -induced Ca^{2+} release from internal stores (Kennedy and Meech, 2002; Lelli et al., 2003; Castellano-Muñoz et al., 2016), making Ca^{2+} clearance from synaptic ribbons even more demanding.

It has been shown that, on stimulation, Ca^{2+} rise in hair cells is spatially confined within $1\ \mu\text{m}$ around synaptic ribbons (Roberts, 1993; Issa and Hudspeth, 1996; Neef et al., 2018), often referred to as Ca^{2+} hotspots, and that Ca^{2+} clearance follows double exponential functions (Tucker and Fettiplace, 1995; Frank et al., 2009). In these studies, Ca^{2+} clearance was sped up when exogenous Ca^{2+} buffer (i.e., EGTA or BAPTA) was applied intracellularly, leading to the two-step hypothesis that the fast and slow components represent Ca^{2+} diffusion via endogenous mobile buffer and Ca^{2+} extrusion through active transport, respectively. However, prolonged voltage steps (20–300 ms) were used in these studies, so that it is not clear how the double exponential clearance would hold up for brief voltage pulses, and if and how it is implicated in phase-locking at the hair cell ribbon synapses. In central synapses without synaptic ribbons, for example, presynaptic Ca^{2+} clearance follows single exponential functions for single spikes (Helmchen et al., 1997; Brenowitz and Regehr, 2007), although double exponential clearance can also be seen in some synapses (Zhang and Linden, 2012; Hamid et al., 2019).

In this study, we took advantage of the split-open preparation of the bullfrog amphibian papilla where phase-locking has been recapitulated in this mature hair cell ribbon synapse through an *in vitro* paired patch-clamp approach (Li et al., 2014). We

Figure 1. Live labeling of hair cell synaptic ribbons in the bullfrog amphibian papilla. **A**, Images of two live hair cells under voltage-clamp, dialyzed with either scrambled (ScRBP) or fRBP at $10\ \mu\text{M}$. Both peptides were tagged with FITC. The cell loaded with ScRBP shows only diffuse fluorescence, whereas the fRBP-loaded cell displays five dense fluorescent puncta attached to the plasma membrane around the basal pole, indicating specific labeling of synaptic ribbons in live hair cells. **B**, Widefield images of a hair cell loaded with fRBP and X-Rhod-5F under patch-clamp. Three and seven synaptic ribbons can be identified in the apical and basal half of the cell, respectively. **C**, Two-photon images of another hair cell loaded with Cy5-RBP and Fluo-4 through the patch pipette. At this focal plane in this particular cell, all nine synaptic ribbons are located in the basal half of the cell. **B, C**, Ca^{2+} entry varies considerably across different synaptic ribbons. **B**, Three synaptic ribbons (#2, #3, and #5) have no noticeable Ca^{2+} entry associated with them. Scale bars: **A–C**, $2\ \mu\text{m}$.

combined this with ultrafast line-scanning two-photon Ca^{2+} imaging to investigate Ca^{2+} clearance from single synaptic ribbons following brief voltage pulses and during phase-locking. We sought to examine the involvement of different Ca^{2+} extrusion mechanisms in Ca^{2+} clearance and phase-locking at the auditory hair cell ribbon synapses.

Materials and Methods

Tissue preparation. All animal care and use followed a protocol reviewed and approved by the University Institutional Animal Care and Use Committee. Adult bullfrogs (*Rana catesbeiana*) of both sexes were killed and decapitated, and the amphibian papillae were dissected in an artificial bicarbonate-based perilymph buffer containing the following (in mM): 95 NaCl, 2 KCl, 2 CaCl_2 , 1 MgCl_2 , 3 D-glucose, 1 creatine, 1 Na-pyruvate, and 25 NaHCO_3 , with pH and osmolarity adjusted to 7.40 and 230 mOsm, respectively. This buffer was also used as the standard external solution for most patch-clamp experiments, unless stated otherwise. The hair cell-containing epithelium was isolated and stretched to expose hair cells and auditory afferent fibers as previously described (Keen and Hudspeth, 2006; Li et al., 2009). Subsequently, the preparation was set in a recording chamber, held in place with an anchor (catalog #64-1419, Warner Instruments), and mounted onto an Olympus BX51WIF microscope in a patch-clamp recording setup. The preparation was perfused at a rate of 2 ml/min with the standard perilymph buffer, continuously bubbled with 95% O_2 and 5% CO_2 . All *in vitro* electrophysiological recording and Ca^{2+} imaging were conducted at room temperature (Chen and von Gersdorff, 2019).

Electrophysiology. Patch-clamp recordings were performed under the whole-cell voltage-clamp mode through an EPC10/2 amplifier (HEKA), and data were acquired with a PC computer running Patchmaster (HEKA). Patch pipettes were pulled from borosilicate

Figure 2. Examining Ca^{2+} extrusion mechanisms in Ca^{2+} clearance from synaptic ribbons in hair cells. **A**, Images of a hair cell loaded with Ca^{2+} indicator Fluo-4 before (top) and during depolarization (bottom), showing that Ca^{2+} signal rises in discrete hotspots, presumably in synaptic ribbons. **B**, Whole-cell Ca^{2+} currents (I_{Ca}) from a hair cell and Ca^{2+} fluorescence changes ($[\text{Ca}^{2+}]_f$) in one synaptic ribbon when the cell was depolarized from the holding potential (i.e., -90 mV) to -30 mV for 100 (red) and 200 ms (black). The longer stimulation evoked a stronger $[\text{Ca}^{2+}]_f$ rise, but on repolarization, $[\text{Ca}^{2+}]_f$ declined rapidly for both stimulations, and both of the $[\text{Ca}^{2+}]_f$ decays are fitted very well to a double exponential function (dashed lines). **C**, Representative $[\text{Ca}^{2+}]_f$ evoked by 200 ms depolarization before (black) and after indicated treatment (red). Treatment of pH 9.0, Ru360, and t-BHQ resulted in a markedly slowed $[\text{Ca}^{2+}]_f$ decay. In contrast, Li^+ treatment had no effect on the $[\text{Ca}^{2+}]_f$ decay. For all experiments, $[\text{Ca}^{2+}]_f$ was normalized to the peak amplitude. Calibration: $0.25 \Delta F/F_{\text{peak}}$, 1 s. **D**, Ratio of the fast component ($\text{Ratio}_{\text{fast}}$), calculated as $A_{\text{fast}}/(A_{\text{fast}} + A_{\text{slow}})$, before and after indicated treatment, with individual experiments depicted in black and averages in red. Statistical significance was assessed with a mixed ANOVA test followed by Dunnett's *post hoc* tests. Data are mean $\pm$ SEM. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. N.S., Not significant ($p > 0.05$).

glass capillaries (catalog #1B150F-4, World Precision Instruments), and they have a typical resistance of 5–10 M Ω when filled with an internal solution containing the following (in mM): 80 Cs-gluconate, 20 CsCl, 10 TEA-Cl, 2 EGTA, 3 Mg-ATP, 0.5 Na-GTP, and 10 HEPES, pH 7.30 (230 mOsm). For experiments concerning blockade of the plasma membrane Ca^{2+} ATPase (PMCA) and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), the baseline was obtained with a HEPES-based perilymph buffer where 25 mM NaHCO_3 was replaced with 48 mM HEPES and NaCl was reduced to 80 mM to match the osmolality. In experiments to inhibit PMCA, the HEPES based-perilymph buffer was adjusted to pH 9.0 with NaOH, and NaCl was further reduced to 60 mM. In experiments to inhibit NCX, NaCl was replaced with equal molar LiCl, and pH was adjusted to 7.40 with CsOH. Both hair

cells and afferent fibers were held at -90 mV between stimulations. For Ca^{2+} imaging experiments, hair cells were depolarized to -30 mV. For phase-locking experiments, sinusoidal voltage commands were generated in Igor Pro 6.0 (WaveMetrics) and applied to hair cells through a template feature in Patchmaster (Li et al., 2014). The hair cell membrane potential was first stepped to -55 mV for 50 ms, and then clamped to a sinusoidal wave of 400 Hz and 20 mV (peak-to-peak amplitude, centered at -55 mV) for 4.8 s (or 1920 cycles). Meanwhile, evoked EPSC responses were recorded in afferent fibers under voltage-clamp. In all experiments, liquid junction potential was calculated as ~ 10 mV and corrected offline.

Ca^{2+} imaging. To verify that Ca^{2+} hotspots coincide with synaptic ribbons in our preparation, we supplemented the pipette solution with 150 μM X-Rhod-5F potassium salt, 10 μM FITC-tagged ribbon-binding peptide (fRBP), and 2 mM reduced glutathione (antioxidant). The design of fRBP and its scrambled version (ScRBP) was adopted from a previous study by Zenisek et al. (2004). To image rapid Ca^{2+} rise and decay in Ca^{2+} hotspots, we included 300 μM Fluo-4 in the pipette solution. For all Ca^{2+} imaging experiments, the pipette solution was allowed to equilibrate with the cytosol for a minimum of 4 min after whole-cell access was obtained.

Widefield Ca^{2+} imaging. Ca^{2+} fluorescence in hair cells was excited with a Xenon light source, which was controlled by a mechanic shutter (Thorlabs) through TTL pulses from the patch-clamp amplifier. Excitation and emission of fluorescent probes were achieved with a band pass filter set in the microscope. Fluorescence images were acquired through a Retiga 2000R CCD Camera (QImaging), primarily focused at regions of hair cells juxtaposed to attached afferent fibers. The ROI was strategically reduced to achieve a frame rate of 54–74 Hz. After experiments, small regions containing single Ca^{2+} hotspots ($0.3 \times 0.3 \mu\text{m}$) were selected to extract Ca^{2+} fluorescence intensity changes in time ($[\text{Ca}^{2+}]_f$). $[\text{Ca}^{2+}]_f$ was analyzed without calibration, with only background subtraction, sampled from regions outside the cell.

Two-photon Ca^{2+} imaging. Ca^{2+} fluorescence was acquired under a Nikon A1 resonant scanning multiphoton microscope (A1MP) through an Apo LWD 25 $\times$ water immersion objective (NA 1.1) with a PC running the Nikon NIS-Elements 5.02 software (Nikon Instruments). Hair cells were patched to be dialyzed with Ca^{2+} indicator Fluo-4 (300 μM) and ribbon-binding peptide (tagged with rhodamine B or Cy5, 10 μM). After cells were equilibrated for 5 min, images were acquired from a ROI of 512×512 pixels with the resolution set by an automated Nyquist criterion. Typically, multiple ribbons can be observed, and a test pulse of 50 ms was applied to the cell to

verify Ca^{2+} influx at the ribbons (see Fig. 3A). Then the ribbon with the brightest Ca^{2+} fluorescence was selected, acquisition was executed in the line scan mode, and a maximum rate of ~ 17 kHz can be reliably achieved. Throughout the experiments, stimulation was carefully kept to a minimum, and the ribbon position was periodically checked. Ca^{2+} fluorescence data were tabulated with the National Institutes of Health ImageJ, $[\text{Ca}^{2+}]_f$ was extracted from a single pixel at the ribbon within the line, and the background was extracted from the average of three consecutive pixels from a vacant region within the line, with a macro written for a Linux emulation software (Cygwin 3.0, www.cygwin.com). $[\text{Ca}^{2+}]_f$ data were loaded into the Igor Pro 6.2 and then background-subtracted. For improved reliability in fitting Ca^{2+} clearance to double

Table 1. Effect of blocking Ca^{2+} extrusion mechanisms on Ca^{2+} clearance from synaptic ribbons^a

Treatment	n	τ_{fast} (ms)	τ_{slow} (ms) ^b	Ratio _{fast}
Baseline	8	27.09 ± 5.14	770.20 ± 134.86	0.75 ± 0.03
Vehicle		28.67 ± 3.17	1253.01 ± 316.20	0.74 ± 0.02
Baseline	6	31.54 ± 7.17	1019.37 ± 115.32	0.74 ± 0.03
pH 9.0		22.56 ± 5.99	3096.39 ± 1045.72	0.46 ± 0.04**
Baseline	4	47.42 ± 1.70	620.09 ± 59.49	0.71 ± 0.03
Vanadate		21.18 ± 4.22	3576.30 ± 1412.60	0.43 ± 0.02**
Baseline	6	42.39 ± 7.75	1173.44 ± 271.78	0.75 ± 0.03
Ru360		36.31 ± 6.97	1529.16 ± 379.32	0.54 ± 0.02*
Baseline	5	35.56 ± 3.24	690.42 ± 27.90	0.68 ± 0.01
KBR		37.29 ± 5.06	1357.06 ± 46.11	0.36 ± 0.02***
Baseline	5	41.38 ± 6.74	1112.94 ± 132.97	0.75 ± 0.01
t-BHQ		32.59 ± 8.45	1744.90 ± 281.55	0.42 ± 0.08**
Baseline	4	57.29 ± 8.06	674.76 ± 25.96	0.74 ± 0.02
CPA		28.85 ± 1.84	1803.58 ± 301.63	0.38 ± 0.02***
Baseline	5	41.96 ± 6.91	570.64 ± 121.75	0.77 ± 0.02
Li^+		53.22 ± 4.16	968.11 ± 165.13	0.75 ± 0.03

^aThe 2 (before and after treatment, within-subject factor) × 8 (treatment types, between-subject factor) mixed ANOVA, Dunnett's *post hoc* test.

^b τ_{slow} was significantly increased within groups, independent of treatment.

* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

exponential functions, $[\text{Ca}^{2+}]_f$ traces were smoothed with a Boxcar algorithm of 11 points, and only smoothed traces are presented, except Figure 3B.

Experimental design and statistical analyses. All experiments were designed to achieve sufficient statistical power, and data were analyzed with a combination of WaveMetrics Igor Pro, Microsoft Excel, and IBM SPSS. For Ca^{2+} imaging, changes in Ca^{2+} fluorescence intensity ($[\text{Ca}^{2+}]_f$) were extracted from raw images with ImageJ (Abramoff et al., 2004), and the $[\text{Ca}^{2+}]_f$ decay was fitted to double exponential functions, yielding two amplitudes (A_{fast} and A_{slow}) and two time constants (τ_{fast} and τ_{slow}). To further quantify Ca^{2+} clearance, we calculated the weight of the fast component (Ratio_{fast}) as $A_{\text{fast}}/(A_{\text{fast}} + A_{\text{slow}})$. For phase-locking, the timing of EPSC events was detected and converted into phases, and the vector strength was calculated, as previously described (Li et al., 2014). For presentation purpose, we added 360 degrees to the phases that are close to 0 degrees (i.e., unwrapping) and then subtracted the averaged phase from all the phases (i.e., offsetting), so that all the phase histograms are plotted in relative phase. For Figures 2D, 4B, and 6, mixed ANOVA tests of 2 (time: before and after treatment, within-subject factor) × 8 or 7 (treatment types: Vehicle, pH 9.0, Vanadate, Ru360, KB-R7935 (KBR), tert-butylhydroquinone (t-BHQ), cyclopiazonic acid (CPA), with or without Li^+ , between-subject factor) were performed, followed by Dunnett's tests on the time effect between the Vehicle and each other treatment. For Figure 7E, F, one-way ANOVA tests were performed, followed by Fisher's least significant difference (LSD) tests or Dunnett's tests. For all statistical tests,

Figure 3. Resolving Ca^{2+} clearance from synaptic ribbons following brief stimulations. **A**, Images of a hair cell under voltage-clamp, showing 3 synaptic ribbons with Ca^{2+} influx on stimulation. Dashed line indicates the line scan through one of the ribbons. **B**, Two-photon line scans of Ca^{2+} fluorescence in response to voltage pulses of 5 ms delivered at 20 Hz (top) and the extracted $[\text{Ca}^{2+}]_f$ trace at the ribbon (bottom). Black and red represent the original and smoothed $[\text{Ca}^{2+}]_f$ traces, respectively. **C**, Smoothed $[\text{Ca}^{2+}]_f$ traces in response to voltage pulses from 1 to 200 ms, showing Ca^{2+} clearance can be resolved for voltage pulses as short as 5 ms. **D**, Time constants (τ_{fast} and τ_{slow}) and amplitudes (A_{fast} and A_{slow}) from double exponential fitting of Ca^{2+} clearance in response to voltage pulses from 5 to 200 ms. As the voltage pulse became longer, neither τ_{fast} nor τ_{slow} changed significantly (Pearson correlation test, $p > 0.05$), A_{fast} increased gradually ($p < 0.05$) while A_{slow} increases dramatically ($p < 0.001$). *** $p < 0.001$. * $p < 0.05$. N.S., Not significant ($p > 0.05$).

Figure 4. The same Ca^{2+} extrusion mechanisms were involved in Ca^{2+} clearance following brief stimulations. **A**, Representative $[\text{Ca}^{2+}]_i$ traces in response to voltage pulses of 5 ms, before (black) and after indicated treatment (red). **B**, $\text{Ratio}_{\text{fast}}$ before and after indicated treatment, showing that the same Ca^{2+} extrusion mechanisms were involved in Ca^{2+} clearance following brief stimulations (mixed ANOVA test followed by Dunnett's *post hoc* tests). *** $p < 0.001$. ** $p < 0.01$.

the confidence level was set as $p < 0.05$, and data are presented as mean $\pm$ SEM throughout the study.

Results

Live imaging of Ca^{2+} in synaptic ribbons of auditory hair cells

In hair cells of various preparations, depolarization evokes Ca^{2+} influx at focal regions along the basal pole, presumably occurring in synaptic ribbons where voltage-gated L-type Ca^{2+} channels are aggregated (Tucker and Fettiplace, 1995; Issa and Hudspeth, 1996; Frank et al., 2009; Wong et al., 2019). To localize synaptic ribbons in live cells, a RBP tagged with fluorescence was often applied intracellularly under patch-clamp (Zenisek et al., 2003; Khimich et al., 2005; Frank et al., 2009; Francis et al., 2011). Adopting this approach, we made patch-clamp recording in hair cells in the split-open preparation of the bullfrog amphibian papilla (Li et al., 2009), and dialyzed them with an internal solution containing either scrambled peptide or RBP (10 μM), conjugated with fluorophore FITC. As shown in Figure 1A, the hair cell dialyzed with the scrambled peptide (ScRBP) showed diffuse labeling throughout the intracellular space, whereas the one with RBP developed punctate aggregations at the basal pole region along the plasma membrane, indicating specific labeling of synaptic

ribbons in live hair cells. Throughout the recording, ScRBP or RBP diffused continuously from the patch pipette into the cell, so that the background fluorescence varied broadly from cell to cell (compare Fig. 1A with Fig. 1B), depending on the cell volume, the access resistance, and the time from break-in.

To localize Ca^{2+} entry simultaneously, we also loaded hair cells with Ca^{2+} indicator X-Rhod-5F (150 μM) and depolarized them from the holding potential (i.e., -90 mV) to -30 mV to introduce Ca^{2+} influx (Fig. 1B). With wide-field microscopy imaging, we identified 48 synaptic ribbons at single focal plane in 10 hair cells, and 39 of them (or 81.3%) were located in the basal half of the cell, consistent with a previous report in the bullfrog saccular hair cells (Zenisek et al., 2003). In later experiments, we switched to two-photon laser scanning microscopy with different fluorophores for RBP and Ca^{2+} (Fig. 1C; 10 μM Cy5-RBP and 300 μM Fluo-4), and we found similar result: among 131 synaptic ribbons identified at single focal plane in 16 hair cells, 110 of them (or 84.0%) were located in the basal half of the cell. Synaptic ribbons apical to the nucleus are common in both mammalian vestibular hair cells (Lysakowski et al., 2011) and nonmammalian hair cells (Zenisek et al., 2003; Schnee et al., 2005; Keen and Hudspeth, 2006; Graydon et al., 2011), but rare in the mammalian cochlea hair cells (Pangršič et al., 2015).

It is worth noting here that, in both widefield and two-photon microscopy (Fig. 1B,C), Ca^{2+} fluorescence varies considerably across different synaptic ribbons, in both intensity (e.g., Fig. 1B, Ribbon #4 and #7) and spatial profile (e.g., Fig. 1C, Ribbon #6 and #8), consistent with findings in the mammalian cochlea hair cells (Frank et al., 2009). Occasionally, we found synaptic ribbons associated with no noticeable Ca^{2+} entry (e.g., Fig. 1B, Ribbon #2, #3, and #5), and the fact we observed more of them in the apical half of the cell seems to suggest they are immature or in process of turning over. However, given that we took images at single focal plane, it is likely that Ca^{2+} entry for some of these synaptic ribbons was simply out of focus. For improved consistency and following precedence (Frank et al., 2009), we always chose bright and confined Ca^{2+} entry sites in the basal pole of the cell for analyzing Ca^{2+} dynamics. In all the cells we examined, such Ca^{2+} entries always occurred in synaptic ribbons with no exception.

Ca^{2+} clearance from synaptic ribbons in hair cells: widefield Ca^{2+} imaging

To examine Ca^{2+} clearance from synaptic ribbons, we loaded hair cells with Fluo-4 (300 μM) through the patch pipette under voltage-clamp, and depolarized the cell to -30 mV for 100 or 200 ms. Depolarization evoked a large Ca^{2+} current (I_{Ca}) with little or no inactivation, which is typical of L-type Ca^{2+} current (Fig. 2B). Simultaneously, Ca^{2+} fluorescence images were acquired continuously before, during, and after the depolarization (Fig. 2A), and Ca^{2+} fluorescence intensity ($[\text{Ca}^{2+}]_i$) was extracted from single Ca^{2+} hotspots and plotted against time

(Fig. 2B). $[\text{Ca}^{2+}]_f$ rose quickly on depolarization and reached a higher level with longer depolarization. However, $[\text{Ca}^{2+}]_f$ fell quickly for both depolarization durations, indicating fast and efficient Ca^{2+} clearance from synaptic ribbons. We fitted the $[\text{Ca}^{2+}]_f$ decay to a double exponential function, which yielded a τ_{fast} , τ_{slow} , and $\text{Ratio}_{\text{fast}}$ of 25.96 ± 4.41 ms, 486.55 ± 129.83 ms, and 0.72 ± 0.03 for depolarization of 100 ms, and 46.61 ± 4.24 ms, 526.53 ± 96.09 ms, and 0.75 ± 0.02 for depolarization of 200 ms (mean $\pm$ SEM). We found no significant difference in τ_{fast} , τ_{slow} , or $\text{Ratio}_{\text{fast}}$ between the two depolarization durations ($p > 0.05$, paired Student's t test, $n = 9$), reinforcing the notion that Ca^{2+} clearance from synaptic ribbons is fast and efficient in hair cells.

To investigate the underlying mechanisms, we focused on four major Ca^{2+} extrusion mechanisms and examined changes in Ca^{2+} clearance with treatments to inhibit them pharmacologically. To inhibit the PMCA, we applied 3 mM vanadate through the patch pipette, or used a HEPES-based artificial perilymph buffer (see the Materials and Methods) and raised the pH to 9.0 (Kobayashi and Tachibana, 1995; Tucker and Fettiplace, 1995; Xu et al., 2000). To inhibit the mitochondrial Ca^{2+} uptake (MCU), 2 nM Ru360 or 5.5 μM KBR was added to the standard artificial perilymph buffer (Matlib et al., 1998; Giarmarco et al., 2017). To inhibit the sarcolemma/endoplasmic reticulum Ca^{2+} ATPase (SERCA), 10 μM t-BHQ, or CPA was applied to the standard artificial perilymph buffer (Kass et al., 1989; Kennedy, 2002). To inhibit the NCX, we substituted Na^+ with Li^+ in the HEPES-based artificial perilymph buffer (Blaustein and Hodgkin, 1969).

Ca^{2+} influx was introduced with depolarization of 200 ms and repeated every 2 min after Ca^{2+} indicator reached equilibrium within the cell. The first three recordings were used to establish baseline of Ca^{2+} clearance kinetics, and experimental data for treatments were obtained between 4 and 6 min after the solution change. In the vehicle-treated hair cells (i.e., no solution change), we found no significant change in either τ_{fast} or $\text{Ratio}_{\text{fast}}$ (Table 1). τ_{slow} did show a significant increase, but this increase of τ_{slow} was also found consistently in all four pharmacological treatments (explanation to follow). Importantly, we found that inhibition of PMCA, MCU, or SERCA reduced the $\text{Ratio}_{\text{fast}}$ of Ca^{2+} clearance significantly, whereas inhibition of NCX did not (Fig. 2C,D; Table 1, mixed ANOVA test followed by Dunnett's *post hoc* tests). In all the treatments, we found no significant changes in τ_{fast} (Table 1).

Figure 5. Recapitulating phase-locking in hair cell ribbon synapses with the paired patch-clamp approach *in vitro*. **A**, A DAPI contrast image showing a hair cell afferent fiber pair subjected to patch-clamp recording. **B**, Representative EPSC responses evoked by sinusoidal voltage commands at two time points (black and red). The hair cell was stepped from the holding potential (i.e., -90 mV) to -55 mV for 50 ms, followed by 1920 cycles of sinusoidal voltage commands at 400 Hz with a peak-to-peak amplitude of 20 mV, centered at -55 mV. **C**, Part of recordings in **B** are shown here with higher temporal resolution. EPSC events from both responses are synchronized with sinusoidal voltage commands (light gray), indicating prominent phase-locking of synaptic vesicle releases from hair cells. **D**, Phase histograms of EPSC events, showing no significant deterioration of phase-locking within a few minutes. These two-phase histograms and those in Figure 6 are plotted in relative phase, from -180 to 180 degrees, in that they were converted from the original phase histograms (shown as insets) with unwrapping (i.e., adding 360 degrees to phases close to 0 degrees), and then offsetting (i.e., subtracting the averaged phase). For each phase histogram, the vector strength (V.S.) was calculated to quantify phase-locking. **E**, Amplitude distributions of EPSC events, showing no significant change in EPSC amplitudes between the two time points.

Ca^{2+} clearance from synaptic ribbons in hair cells: two-photon Ca^{2+} imaging

While the camera-based widefield Ca^{2+} imaging experiments described above are quantitative and useful, it comes with two drawbacks. First, the frame rate is slow (<100 Hz) so that the speed of Ca^{2+} clearance, particularly the fast component, could be underestimated. Second, we observed a steady and significant slowing down of the slow component over time (Table 1). This was likely caused by intracellular energy depletion in the whole-cell recording mode and/or phototoxicity when all Ca^{2+} indicator molecules within the cell were constantly excited during

Figure 6. Revealing the involvement of different Ca^{2+} extrusion mechanisms in the phase-locking of exocytosis in auditory hair cells. **A**, Representative phase histograms of EPSC events evoked by sinusoidal voltage commands, showing stable phase-locking when no treatment was applied. The phase histograms are plotted in relative phase after unwrapping and off-setting were applied to the original phases, as shown in Figure 5D. Pooled values of the vector strength (V.S.) calculated from individual experiments (black) and their averages (red) are plotted on the right. **B–E**, Same as in **A**, with indicated treatment. V.S. was significantly reduced in pH 9.0 and vanadate (**B**), Ru360 and KBR (**C**), and t-BHQ and CPA (**D**), indicating that PMCA, MCU, and SERCA are required for phase-locking. V.S. was not changed in the treatment of Li^+ (**E**), suggesting that NCX is not involved in the phase-locking of exocytosis in hair cells (mixed ANOVA test followed by Dunnett's *post hoc* tests). ** $p < 0.01$. * $p < 0.05$. N.S., Not significant ($p > 0.05$).

imaging (Hoebé et al., 2007). Because this happened independent of pharmacological treatment, it is impossible to evaluate change of the slow component following the blockade of Ca^{2+} extrusion mechanisms with pharmacological treatments. To overcome these two drawbacks, we took a complementary approach of two-photon Ca^{2+} imaging with line scan, which minimizes excitation of Ca^{2+} indicator and offers a sampling rate of ~ 17 kHz (Fig. 3A,B). Given the unbinding rate of 210 s^{-1} for Fluo-4 (Tang et al., 2015), this sampling rate is quite excessive, but it afforded us the ability to average and thus remove stochastic noise in the signal, revealing small and subtle changes in $[\text{Ca}^{2+}]_f$. As shown in Figure 3C, the $[\text{Ca}^{2+}]_f$ rise is noticeable for voltage pulses as short as 2 ms. However, the $[\text{Ca}^{2+}]_f$ decay can be resolved with curve fitting only for voltage pulses of 5 ms and longer. We fitted the $[\text{Ca}^{2+}]_f$ decay to a

double exponential function, and we found that, as the stimulation became longer from 5–200 ms, the amplitudes of both the fast and slow component (A_{fast} and A_{slow}) increased significantly (Pearson correlation test, $p < 0.05$ and $p < 0.001$ for A_{fast} and A_{slow} , respectively). Furthermore, because A_{slow} increased more than A_{fast} , $\text{Ratio}_{\text{fast}}$ decreased significantly ($p < 0.001$, data not shown); therefore, the overall speed of Ca^{2+} clearance became slower. Remarkably, neither of the two time constants (τ_{fast} and τ_{slow}) changed significantly ($p > 0.05$; see Fig. 3D). Together, these results indicate that, although the overall speed of Ca^{2+} clearance becomes slower for longer stimulation, the fast component of Ca^{2+} clearance remains largely intact, revealing and highlighting the remarkable speed and efficiency of Ca^{2+} clearance from synaptic ribbons in hair cells.

To further examine the involvement of different Ca^{2+} extrusion mechanisms, we decided to focus on Ca^{2+} clearance following brief voltage pulses of 5 ms, which are more physiological (i.e., more similar to brief voltage changes seen in hair cells *in vivo*) compared with long step depolarizations of 200 ms. Consistent with the wide-field Ca^{2+} imaging approach, we found that $\text{Ratio}_{\text{fast}}$ was significantly reduced when PMCA, MCU, or SERCA was inhibited pharmacologically (Fig. 4A,B; mixed ANOVA test followed by Dunnett's *post hoc* tests). In all the treatments, we found no significant changes in either τ_{fast} or τ_{slow} (data not shown).

Inhibition of Ca^{2+} extrusion disrupts phase-locking of exocytosis in hair cells

We have shown that inhibition of Ca^{2+} extrusion slows down Ca^{2+} clearance from synaptic ribbons in hair cells. Next, we wanted to investigate if and how the phase-locking of synaptic vesicle releases from hair cells is affected. As described previously (Li et al., 2014), we performed paired patch-clamp recording in hair cell-afferent fiber pairs (Fig. 5A), depolarized the hair cell from the holding potential (i.e., -90 mV) to -55 mV to mimic the resting membrane potential of hair cells *in vivo*. We then applied sinusoidal voltage commands at 400 Hz with a peak-to-peak amplitude of 20 mV and centered at -55 mV . Afferent fibers were held at -90 mV and evoked EPSC responses were recorded (Fig. 5B). As previously demonstrated (Li et al., 2014), the timing of individual EPSC events showed prominent synchrony with the sinusoidal stimulation cycles (Fig. 5C), an indication of phase-locking. To quantify phase-locking, the timing of EPSC events was converted into phases according to the sinusoidal stimulation. Based on these phases, we constructed period histograms and calculated the vector strength (Fig. 5D). In vehicle-treated hair cells (i.e., no solution change), we found that the phase-locking of EPSC events was stable

within a few minutes (Fig. 6A). Importantly, we found that the inhibition of PMCA, MCU, or SERCA reduced the phase-locking of EPSC events significantly, while no significant change was found in the inhibition of NCX (Fig. 6; Table 2; mixed ANOVA test followed by Dunnett's *post hoc* tests), in close agreement with the findings on Ca^{2+} clearance described above. For all experiments, no significant difference was found in the number or amplitude of EPSC events during phase-locking (Fig. 5E; Table 2).

To reveal the underlying mechanisms for phase-locking disruption, we performed two-photon Ca^{2+} imaging in synaptic ribbons while applying sinusoidal voltage stimulation to hair cells (Fig. 7A). While the sampling rate for $[\text{Ca}^{2+}]_f$ (~ 17 kHz) is sufficient for the stimulation of 400 Hz, the rise and decay of $[\text{Ca}^{2+}]_f$ were too subtle to be resolved on cycle-by-cycle basis (Fig. 7B). This is expected because based on the unbinding rate of 210 s^{-1} for Fluo-4 (Tang et al., 2015), $[\text{Ca}^{2+}]_f$ changes within a sinusoidal cycle (2.5 ms) cannot be sufficiently resolved. However, it is noticeable that $[\text{Ca}^{2+}]_f$ fluctuated more when the sinusoidal voltage commands were on (Fig. 7A). Upon closer examination, we found that the root mean square (RMS, a parameter for signal fluctuation) of $[\text{Ca}^{2+}]_f$ was significantly increased when sinusoidal stimulation was turned on ($p < 0.001$, paired Student's *t* test, $n = 7$; Fig. 7C). In contrast, the RMS of $[\text{Ca}^{2+}]_f$ did not change significantly when step depolarization was turned on ($p > 0.05$). This suggests that the RMS of $[\text{Ca}^{2+}]_f$ during sinusoidal stimulation reflects, at least partially, an averaged amplitude of the Ca^{2+} signal within sinusoidal cycles.

We then applied treatments to inhibit different Ca^{2+} extrusion mechanisms, and we found that the RMS of $[\text{Ca}^{2+}]_f$ during sinusoidal stimulation was significantly increased by inhibition of PMCA, MCU, or SERCA compared with the control (Fig. 7D,E), consistent with their effects on Ca^{2+} clearance and phase-locking described above. The larger change in the RMS of $[\text{Ca}^{2+}]_f$ for the pH 9 treatment could be caused by Ca^{2+} channel relief from proton block in alkaline solution (Cho and von Gersdorff, 2014). In addition, we examined changes in the basal $[\text{Ca}^{2+}]_f$ at rest before stimulation, and we found it was also significantly increased by inhibition of PMCA, MCU, or SERCA (one-way ANOVA test followed by Fisher's LSD tests; Fig. 7F). Together, these results suggest that inhibition of Ca^{2+} extrusion raises the basal Ca^{2+} level at resting and increases Ca^{2+} influx during sinusoidal stimulation, both of which are likely to overwhelm the exocytosis machinery in synaptic ribbons and cause disruption of phase-locking at the hair cell ribbon synapses (see more on this in Discussion).

Discussion

In this study, we combined paired patch-clamp recording and two-photon Ca^{2+} imaging and investigated Ca^{2+} clearance from synaptic ribbons in mature hair cells from the bullfrog amphibian papilla. We found that Ca^{2+} clearance follows a double exponential function, even for brief stimulations, and that treatments inhibiting Ca^{2+} extrusion slow down the clearance and disrupt the phase-locking of exocytosis from auditory hair cells.

Ca^{2+} clearance in hair cells: speed and efficiency

In central synapses without synaptic ribbons, Ca^{2+} clearance in response to single spikes follows a single exponential function, and the time constant (τ) varies from 20 to >100 ms, even for synapses from the same axon (Brenowitz and Regehr, 2007). In

Table 2. Effect of blocking Ca^{2+} extrusion mechanisms on phase-locking of EPSC events^a

Treatment	<i>n</i>	Vector strength	No. of EPSC events	Mean amplitude of EPSC events
Baseline	6	0.62 ± 0.04	929.17 ± 187.27	119.46 ± 16.67
Vehicle		0.57 ± 0.05	864.67 ± 151.41	111.29 ± 16.79
Baseline	7	0.56 ± 0.04	1164.86 ± 256.34	135.21 ± 15.38
pH 9.0		$0.26 \pm 0.06^*$	1276.71 ± 246.86	132.21 ± 17.64
Baseline	5	0.46 ± 0.06	1602.20 ± 55.05	116.89 ± 9.76
Vanadate		$0.31 \pm 0.05^*$	1365.20 ± 83.20	100.77 ± 12.66
Baseline	7	0.55 ± 0.04	1459.86 ± 236.07	114.75 ± 10.42
Ru360		$0.22 \pm 0.05^{**}$	1236.43 ± 198.60	98.22 ± 7.51
Baseline	5	0.53 ± 0.07	1348.80 ± 165.06	125.36 ± 17.74
KBR		$0.22 \pm 0.06^*$	1094.60 ± 200.02	125.08 ± 20.54
Baseline	5	0.49 ± 0.04	1152.20 ± 272.28	101.32 ± 17.08
t-BHQ		$0.16 \pm 0.04^{**}$	818.60 ± 158.37	100.64 ± 10.05
Baseline	4	0.52 ± 0.06	1190.50 ± 288.80	120.09 ± 22.66
CPA		$0.23 \pm 0.07^*$	1082.75 ± 289.71	81.90 ± 16.01
Baseline	6	0.61 ± 0.04	1000.00 ± 259.71	130.40 ± 10.81
Li^+		0.59 ± 0.04	1123.83 ± 236.36	109.43 ± 6.53

^aThe 2 (before and after treatment, within-subject factor) $\times$ 8 (treatment types, between-subject factor) mixed ANOVA, Dunnett's *post hoc* test. * $p < 0.05$. ** $p < 0.01$.

response to multiple spikes, Ca^{2+} clearance becomes slower and in some synapses follows a double exponential function, but the time constant for the fast component (τ_{fast}) is still >20 ms (Zhang and Linden, 2012). In contrast, we found in this study that Ca^{2+} clearance from synaptic ribbons in hair cells follows a double exponential function with a τ_{fast} of ~ 6 ms, and the double exponential clearance occurs even for stimulation as short as 5 ms (Fig. 3). Given the slow unbinding rate of the Ca^{2+} indicator in our experiments, τ_{fast} is likely to be underestimated considerably, so that the real τ_{fast} of Ca^{2+} clearance is even faster, as computer simulation has shown (Bortolozzi et al., 2008; Hamid et al., 2019). What is even more remarkable is that as the stimulation grows longer from 5 to 200 ms, the weight of the fast component (Ratio_{fast}) decreases, but neither τ_{fast} nor τ_{slow} changes significantly, indicating that these hair cells can handle 40 times more Ca^{2+} load with equal speed and efficiency.

Ca^{2+} clearance in hair cells: mechanisms

The fast and slow component of the double exponential Ca^{2+} clearance in hair cells has been attributed to diffusion mediated by endogenous mobile Ca^{2+} buffer, and extrusion mediated by active Ca^{2+} transport, respectively (Tucker and Fettiplace, 1995). This is well supported by the observation that exogenous mobile Ca^{2+} buffer (i.e., EGTA or BAPTA) applied intracellularly speeds up Ca^{2+} clearance in hair cells (Frank et al., 2009). Consistently, small and mobile Ca^{2+} binding proteins, such as parvalbumin, calretinin, and calbindin, are abundantly expressed in frog and mouse auditory hair cells (Edmonds et al., 2000; Heller et al., 2002; Quiñones et al., 2012; Pangršič et al., 2015). Interestingly, for central synapses, additional exogenous Ca^{2+} mobile buffer slows down Ca^{2+} clearance (Helmchen et al., 1997). This striking difference is likely because of the difference in surface-area-to-volume ratio. In a confined volume as often seen in central synapses, mobile Ca^{2+} buffer can bind and remove free Ca^{2+} ; but because it is bound to stay local, it also serves as a reserve to release Ca^{2+} . For hair cells, however, mobile Ca^{2+} buffer can bind Ca^{2+} and diffuse away. Therefore, the large intracellular volume that synaptic ribbons are facing serves a purpose of speeding up Ca^{2+} clearance in hair cells.

Figure 7. Blocking Ca^{2+} extrusion increased the amplitude of Ca^{2+} signal in response to sinusoidal voltage commands and raised the basal Ca^{2+} level at rest. **A**, Representative $[\text{Ca}^{2+}]_i$ trace (black) in response to an episode of sinusoidal voltage commands (gray). **B**, Representative $[\text{Ca}^{2+}]_i$ measurements during sinusoidal stimulation, showing that no Ca^{2+} rise and fall can be discerned within sinusoidal cycles. **C**, RMS (a parameter for signal fluctuation) of $[\text{Ca}^{2+}]_i$ during the holding membrane potential (i.e., -90 mV, Rest), the step depolarization (i.e., -55 mV, Step), and the sinusoidal voltage commands (Sine, see the stimulation template in **A**). Step depolarization failed to change RMS significantly ($p > 0.05$), but turning on sinusoidal stimulation increased RMS significantly ($p < 0.001$, paired Student's t test, $n = 7$). **D**, Representative $[\text{Ca}^{2+}]_i$ traces in response to sinusoidal voltage commands, with no treatment (Vehicle) and the application of KBR. **E**, Change of RMS in $[\text{Ca}^{2+}]_i$ during sinusoidal voltage stimulation before and after treatment indicated, showing that blocking Ca^{2+} extrusion mechanisms increased the amplitude of Ca^{2+} signal in response to sinusoidal voltage commands (one-way ANOVA test followed by Dunnett's *post hoc* tests). **F**, Normalized change in the basal $[\text{Ca}^{2+}]_i$ level before and after indicated treatment, showing that blocking Ca^{2+} extrusion significantly raised the basal Ca^{2+} level in synaptic ribbons at rest (one-way ANOVA test followed by Fisher's LSD tests). *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. n.s., Not significant ($p > 0.05$).

In addition to diffusion, the fast and efficient Ca^{2+} clearance in hair cells requires active Ca^{2+} transport to remove Ca^{2+} out of the intracellular space. Indeed, we found the coinvolvement of three primary Ca^{2+} extrusion mechanisms (the PMCA, the MCU, and the SERCA), but not the secondary mechanism (the NCX). This turns out to be unique to the hair cell ribbon synapses. In the calyx of Held synapse, a central synapse in the auditory brainstem where temporal precision is also in high demand, only PMCA and MCU are involved in the Ca^{2+} clearance from the presynaptic terminal, supplemented with a K^{+} -dependent form of NCX (Billups and Forsythe, 2002; Kim et al., 2005). In the terminal of retinal photoreceptors, inhibition of NCX had no effect on Ca^{2+} clearance, whereas PMCA was identified as the major Ca^{2+} extrusion mechanism. However, PMCA expression seems to be distant from synaptic ribbons (Morgans et al., 1998), perhaps because phase-locking to visual signals only needs to

follow-up to ~ 50 Hz (i.e., flicker-fusion frequency). Similarly, in the retinal bipolar cell terminal, inhibition of NCX showed effect only for extended Ca^{2+} influx evoked by long depolarization, whereas inhibition of PMCA caused significant Ca^{2+} buildup in the synaptic terminal (Kobayashi and Tachibana, 1995).

Ca^{2+} clearance in hair cells: implication in phase-locking

Earlier studies of Ca^{2+} clearance in hair cells were conducted with single voltage pulses of 20–200 ms (Tucker and Fettiplace, 1995; Frank et al., 2009), so that it is not clear whether the double exponential Ca^{2+} clearance would hold up for short voltage pulses that are more physiological, and how the fast and slow component would be implicated in phase-locking. We found that the weight of the slow component decreased and even diminished as the voltage pulse became as short as 5 ms (Fig. 3D). Therefore, while extrusion may contribute significantly for phase-locking at very low frequencies (e.g., < 50 Hz), diffusion is likely to be the primary mechanism for Ca^{2+} clearance in phase-locking, especially for high frequencies. Coincidentally, this dominance of diffusion was also uncovered recently in the hippocampal CA1 neurons (Hamid et al., 2019).

Although extrusion is too slow to directly contribute to Ca^{2+} clearance within sinusoidal cycles during phase-locking, we found that its inhibition disrupted phase-locking, indicating that it still plays a critical role. It is conceivable that diffusion and extrusion are interconnected dynamically, so that extrusion contributes indirectly by modulating diffusion. Indeed, it has been shown that exogenous mobile Ca^{2+} buffer speeds up both components (Frank et al., 2009), and that blocking Ca^{2+} extrusion diminishes both components (Tucker and Fettiplace, 1995). In the present study, we found that, along with the basal Ca^{2+} rise at rest following inhibition of different Ca^{2+} extrusion mechanisms (Fig. 7F), the RMS of Ca^{2+} signal during sinusoidal stimulation was also increased (Fig. 7E), suggesting that Ca^{2+} level is elevated during stimulation. It is likely that the basal Ca^{2+} rise reduces the Ca^{2+} concentration gradient that drives diffusion, which slows down the fast clearance of Ca^{2+} from synaptic ribbons and increases the Ca^{2+} level during stimulation.

Release of synaptic vesicles is tightly regulated in ribbon synapses, and excessive Ca^{2+} could cause asynchronized release of synaptic vesicles (Singer and Diamond, 2003). The basal Ca^{2+} rise at rest and the elevated level of Ca^{2+} during stimulation could work independently or synergistically to bring excessive Ca^{2+} to synaptic ribbons and therefore disrupt phase-locking, by causing additional releases of synaptic vesicles and/or changing the timing of existing ones. However, with the inhibition of the same Ca^{2+} extrusion mechanisms, we observed no increase in the number of EPSCs in response to sinusoidal stimulation (Table 2), suggesting that the excessive Ca^{2+} within and between stimulations does not cause additional releases of synaptic

vesicles. Therefore, it is likely that it disrupts phase-locking by changing timing of existing releases.

It is worth noting here that, unlike the single ribbon active zone found in the mammalian inner hair cell synapses, each bullfrog hair cell-afferent fiber pair in our preparation contains multiple ribbon synapses (Keen and Hudspeth, 2006; Graydon et al., 2014), which is often seen in nonmammalian hearing organs (Schnee et al., 2005). Consequentially, afferent fibers in nonmammalian hearing organs receive more frequent releases of synaptic vesicles, and this has been shown for both step depolarization (Goutman and Glowatzki, 2007; Li et al., 2009) and more physiological stimulations (Goutman, 2012; Li et al., 2014). However, almost all releases of synaptic vesicles are capable to trigger spikes in the mammalian inner hair cell synapses (Rutherford et al., 2012), whereas only a small percentage of them do so in nonmammalian hearing organs (Schnee et al., 2013; Li et al., 2014), a difference that can be attributed to different volumes and input resistances in bouton and calyx terminals (Chen and von Gersdorff, 2019). Taking these two countering differences together, afferent fibers in both categories would have sufficient synaptic drive to fire frequent spikes for phase-locking. But overall, phase-locking in nonmammalian hearing organs could be less precise because spikes have more jitter when they are triggered by multiple overlapping synaptic releases rather than by single ones. To partially make up for this reduced precision, at least in our preparation, more precision is strategically assigned to large and multivesicular releases that are more likely to trigger spikes (Li et al., 2014). Nevertheless, given that the double exponential Ca^{2+} clearance from synaptic ribbons is found in hair cells of mice, frogs, and turtles (Tucker and Fettiplace, 1995; Rispoli et al., 2001; Bortolozzi et al., 2008; Frank et al., 2009), the results presented in this study with mature bullfrogs are likely to contribute substantially to our knowledge on the subject of Ca^{2+} buildup and clearance in auditory hair cells.

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