Brief Communications

Actin Filaments Regulate Exocytosis at the Hair Cell Ribbon Synapse

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Exocytosis at the inner hair cell ribbon synapse is achieved through the functional coupling between calcium channels and glutamate-filled synaptic vesicles. Using membrane capacitance measurements, we investigated whether the actin network regulates the exocytosis of synaptic vesicles at the mouse auditory hair cell. Our results suggest that actin network disruption increases exocytosis and that actin filaments may spatially organize a subfraction of synaptic vesicles with respect to the calcium channels.

Key words: **cochlea; membrane capacitance; patch-clamp; synaptic release**

Significance Statement

Inner hair cells (IHCs), the auditory sensory cells of the cochlea, release glutamate onto the afferent auditory nerve fibers to encode sound stimulation. To achieve this task, the IHC relies on the recruitment of glutamate-filled vesicles that can be located in close vicinity to the calcium channels or more remotely from them. The molecular determinants responsible for organizing these vesicle pools are not fully identified. Using pharmacological tools in combination with membrane capacitance measurements, we show that actin filament disruption increases exocytosis in IHCs and that actin filaments most likely position a fraction of vesicles away from the calcium channels.

Introduction

Transmitter release at the first auditory synapse is a fundamental step in transducing acoustic stimulation into a neural message. Incoming acoustic stimulation depolarizes the auditory sensory hair cells, the inner hair cells (IHCs), to elicit calcium influx through voltage-gated calcium channels so that glutamate-filled synaptic vesicles can be released into the synaptic cleft. Activation of postsynaptic glutamate receptors enable the afferent auditory fibers to convey the neural message to the auditory nuclei (Nouvian et al., 2006). Therefore, the synaptic transfer relies on the organization and recruitment of synaptic vesicles with respect to the calcium channels (Brandt et al., 2005; Frank et al., 2010; Wong et al., 2014). Two pools of synaptic vesicles operate at the hair cell ribbon synapse, as shown by presynaptic membrane capacitance measurements and EPSC recordings: the readily releasable pool (RRP), which reflects the fusion of the synaptic vesicles in the vicinity of the calcium channels, and the sustained releasable pool (SRP), which corresponds to the exocytosis of remote vesicles and/or to the resupply of the RRP (Moser and Beutner, 2000; Spassova et al., 2004; Johnson et al., 2005; Schnee et al., 2005; Rutherford and Roberts, 2006; Goutman and Glowatzki, 2007; Li et al., 2009). To date, only the multiple C2-domain otoferlin protein has been shown to regulate the replenishment of the RRP by the SRP in IHCs. In the Pachanga mutant mouse, RRP exocytosis probed by short depolarization is not altered, whereas SRP probed by longer depolarization is gradually affected with the reduction of otoferlin expression (Pangršič et al., 2010). Among the candidates that modulate the synaptic vesicle supply in the nervous system's synapses, actin filaments are known to have a dual role. For example, disruption of actin network increases the frequency of EPSCs in hippocampal neurons (Morales et al., 2000). In contrast, depolymerization of actin filaments (F-actin) slows the vesicle replenishment in the calyx of Held synapse (Sakaba and Neher, 2003). Alternatively, synaptic vesicle exocytosis can be completely independent of F-actin (Holt et al., 2003). Although cytoskeleton proteins such as actin are localized near the synaptic ribbon in bullfrog hair cells (Graydon et al., 2011), the role of actin on synaptic vesicle exocytosis at the

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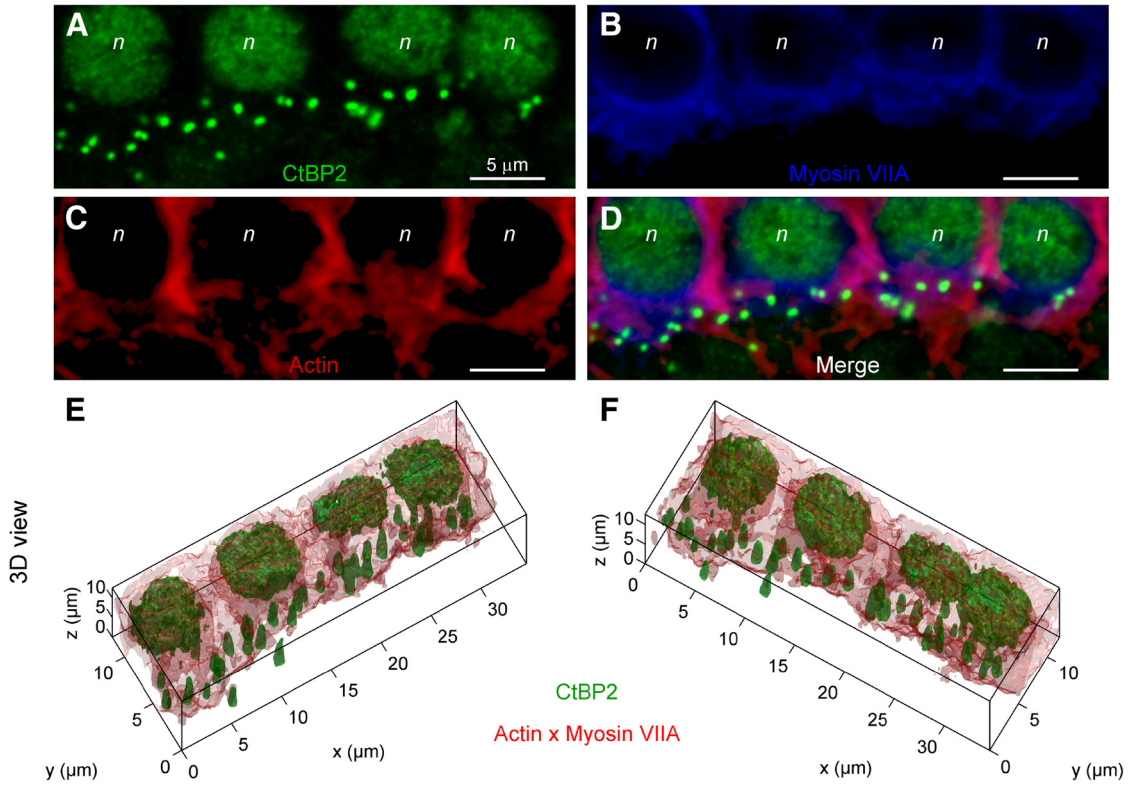

Figure 1. Expression of actin in IHCs. ***A–D***, Single confocal section through four neighboring IHCs. ***A***, Synaptic ribbons were stained with antibody against CtBP2 (green). Note that the IHC nucleus is also labeled due to the nuclear expression of CtBP2. ***B***, IHCs were labeled with anti-myosin VIIA antibody (blue). ***C***, Actin was visualized with rhodamine-conjugated phalloidin (red). ***D***, Merged image revealing that actin is localized thoughout the cytoplasm. n, Nucleus. Scale bar, 5 μm. ***E***, ***F***, Isosurface 3D reconstruction using the stack of 2D confocal images (two viewpoints: E, azimuth = −30°, elevation = 70°; F, azimuth = +30°, elevation = 70°). Green, CtBP2/RIBEYE immunofluorescence; red, colocalization of actin and myosin VIIA.

hair cell ribbon synapse has never been determined. Taking advantage of drugs that are known to depolymerize actin filaments, we show that actin regulates the organization of a fraction of vesicles at the hair cell ribbon synapse.

Materials and Methods

Animals. Swiss mice of either sex were bred and handled following the animal welfare guidelines of the Institut National de la Santé et de la Recherche Médicale (Inserm) and approved by the Ministère Français de l'Agriculture et de la Pêche (authorization #A3417231).

Immunohistochemistry. Immunohistochemistry and confocal microscopy (Leica TCS SP8-UV) of apical turns were performed as described previously (Sendin et al., 2007) with mouse IgG anti C-terminal binding protein 2 (CtBP2; 1:1000; BD Biosciences) and rabbit polyclonal IgG anti myosin VIIA (1:300; Proteus Biosciences) and the secondary antibodies Alexa Fluor 488-labeled donkey IgG anti-mouse (1:1000; Invitrogen) and Alexa Fluor 647-labeled donkey IgG anti-rabbit (1:1000; Invitrogen). To label F-actin in the cuticular plate and the basolateral membrane, we used phalloidin TRITC (1:1000; Invitrogen).

Electrophysiology. Patch-clamp of IHCs (postnatal day 14–30) from apical coils of freshly dissected organs of Corti were performed as described previously (Nouvian et al., 2011). The extracellular solution contained the following (in mM): 105 NaCl, 35 tetraethylammonium-Cl (TEA-Cl), 2.8 KCl, 1 $MgCl_2$, 10 HEPES, 1 CsCl, 2 $CaCl_2$, and 10 D-glucose. The pipette solution for whole-cell experiments contained the following (in mM): 135 Cs-glutamate, 10 TEA-Cl, 10 HEPES, 1 $MgCl_2$, 10 4-aminopyridine, 2 Mg-ATP, 0.3 Na-GTP, and 0.1 EGTA. All solutions were adjusted to pH 7.2 and had osmolarities between 290 and 310 mosmol/L. All chemicals were obtained from Sigma-Aldrich. Latrunculin A, cytochalasin D, and phalloidin were dissolved in DMSO, the final concentration of which in the intracellular solution was 0.25–0.75%. Ca^{2+}-triggered exocytosis was probed after a 10 min infusion into IHCs of 20 μM latrunculin A, 20 μM cytochalasin D, or 100 μM phalloidin via the patch-pipette. For control cells, the intracellular solution contained the corresponding DMSO concentration (0.5% for latrunculin A and cytochalasin D, 0.25% for phalloidin, and 0.75% for latrunculin A plus phalloidin). Control and drug-treated cells were probed over the same time frame for better comparison. Except for the exocytosis amplitude plots against the duration of stimulation (see Fig. 4), all of the controls were pooled together. An EPC-10 amplifier (HEKA Elektronik) controlled by Patchmaster software (HEKA Elektronik) was used for all measurements as described previously (Nouvian et al., 2011). Membrane capacitance jump (ΔC_m) was estimated as the difference of the mean C_m over 400 ms after the end of the depolarization (the initial 250 ms were skipped) and the mean prepulse capacitance (400 ms). Data analysis was performed using Igor Pro software (Wave-Metrics). Means are expressed as ±SEM and were compared by Wilcoxon test.

Results

Actin distribution at the IHC basolateral side

Although the abundant distribution of actin at the apical side of the hair cell—that is, in the stereocilia bundle and the cuticular plate—is well documented, its distribution at the basolateral side of the hair cells has received little attention (Furness et al., 2005). Phalloidin–rhodamin staining and myosin VIIA immunofluorescence show that some actin filaments run below the nucleus into the hair cells' basolateral side (Fig. 1). Although F-actin localized nearby the synaptic area in our confocal microscopy observations, we did not detect enrichment near the synaptic ribbons labeled with anti-CtBP2 antibody. Together with the large expression of actin at

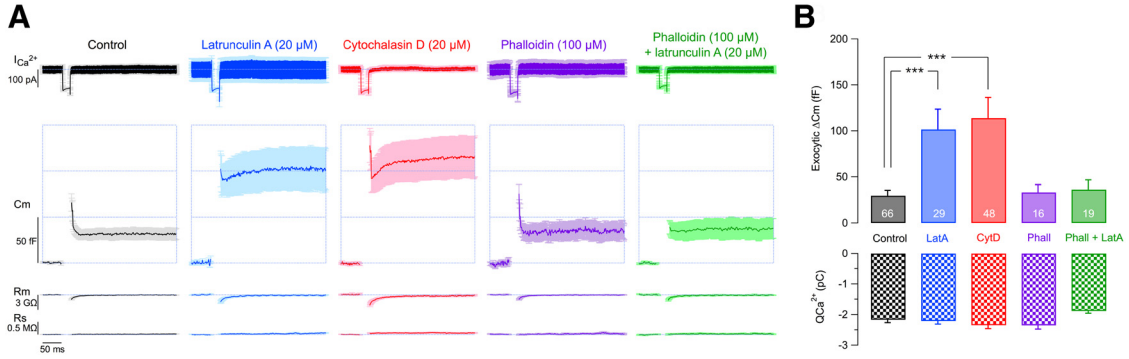

Figure 2. Actin network depolymerization increases exocytosis. ***A***, Whole-cell Ca^{2+} current (ICa^{2+}), membrane capacitance (C_m), membrane resistance (R_m), and series resistance (R_s) average traces. Data were recorded from IHCs after 10 min infusion with 20 μM latrunculin A, 20 μM cytochalasin D, 100 μM phalloidin, or 100 μM phalloidin plus 20 μM latrunculin A through the patch-pipette. Calcium-triggered exocytosis was evoked in response to a single 20 ms depolarization to −27 mV from a holding potential of −87 mV. ***B***, Average ΔC_m (top) and Ca^{2+} charges (bottom) evoked by 20 ms depolarization after 10 min infusion of latrunculin A (LatA, 20 μM), cytochalasin D (CytD, 20 μM), phalloidin (Phall, 100 μM), or phalloidin plus latrunculin A (Phall, 100 μM + LatA, 20 μM). All of the controls (black) are pooled together. Only one depolarization step was evoked per IHC and the numbers of IHCs recorded are indicated in white. ***$p < 0.001$.

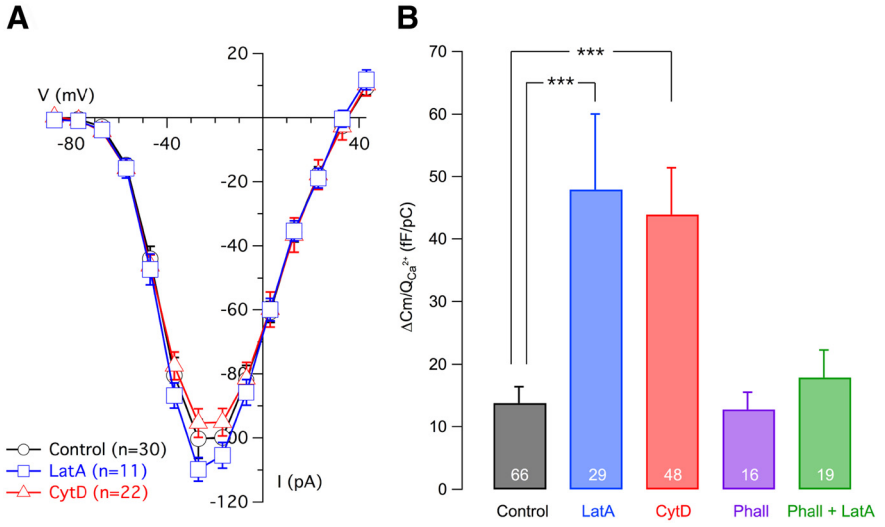

Figure 3. Actin network depolymerization does not alter IHC calcium influx. ***A***, Ca^{2+} current I/V relationships of IHCs infused for 10 min with latrunculin A (LatA, 20 μM) and cytochalasin D (CytD, 20 μM). ***B***, Release efficiency of IHCs treated with latrunculin A (LatA, 20 μM), cytochalasin D (CytD, 20 μM), phalloidin (Phall, 100 μM), and phalloidin plus latrunculin A (Phall, 100 μM + LatA, 20 μM). Release efficiency was measured as the $\Delta C_m/QCa^{2+}$ ratio from Figure 2*B*. ***$p < 0.001$.

the apical side, this result suggests that actin filaments also populate the basolateral side of the hair cells.

Actin depolymerization increases exocytosis

The regulation of calcium-triggered exocytosis by the actin network was investigated using drugs that are known to depolymerize actin filaments. We first infused latrunculin A, a drug that sequesters the G-actin monomer and therefore leads to the depolymerization of actin, into hair cells for 10 min. In response to a 20 ms depolarizing pulse to −27 mV, infusion of 20 μM latrunculin A increased exocytosis, probed by capacitance measurements as a proxy of synaptic vesicle fusion (Fig. 2; 29.6 ± 5.7 fF for control cells vs 101.5 ± 22.1 fF for latrunculin A-treated cells; $p < 0.001$). To confirm these results, we then probed the effect of cytochalasin D, which caps the barbed end of F-actin to depolymerize the actin filaments (Fig. 2). Here again, we found out that infusion of 20 μM cytochalasin D for 10 min increased the synaptic vesicle exocytosis evoked by the 20 ms depolarization step (29.6 ± 5.7 fF vs 113.8 ± 22.5 fF for control and cytochalasin D-treated cells, respectively; $p <$ 0.001). Although 100 μM phalloidin, which stabilizes actin, alone did not significantly affect IHC secretion (33.2 ± 8.5 fF), coapplication of 20 μM latrunculin A together with 100 μM phalloidin (36.3 ± 10.4 fF) prevented the increase of synaptic vesicle exocytosis elicited by latrunculin A alone (Fig. 2). Together, these effects suggest that the actin filament network regulates neurotransmitter release at the hair cell ribbon synapse.

Actin depolymerization does not alter the calcium current amplitude

The facilitating effect of actin depolymerization may result from an increase of the calcium current. However, the calcium current amplitude did not show any significant difference between control and latrunculin A- or cytochalasin D-treated cells (calcium peak current −100.3 ± 6.1 pA, −109.8 ± 3.7 and −95.4 ± 4.5 pA for control, latrunculin A, and cytochalasin D, respectively; Fig. 3*A*). In addition, a higher release efficiency given by the ratio of the capacitance jump over the corresponding calcium charge was observed in cells exposed to latrunculin A as well as cytochalasin D (Fig. 3*B*; 13.8 ± 2.6 fF/QCa^{2+} vs 47.9 ± 12.1 fF/QCa^{2+} and 43.9 ± 7.5 fF/QCa^{2+} for control, latrunculin A and cytochalasin-treated cells, respectively; $p < 0.001$).

Restricted effect of actin depolymerization on exocytosis

Next, we investigated whether actin filament network disruption also affects SRP or the RRP–SRP relationship. To do so, we probed exocytosis evoked by steps of different duration because short durations of stimulation mostly recruit exocytosis of the RRP, whereas longer pulses mobilize mainly the synaptic vesicles belonging to the SRP (Nouvian et al., 2006). The exocytosis plot against the time duration of the stimulation shows that latrunculin A (20 μM) or cytochalasin D (20 μM) mainly affected exocytosis evoked by a 20 ms step depolarization, leaving the secretion elicited by shorter (5 ms, con-

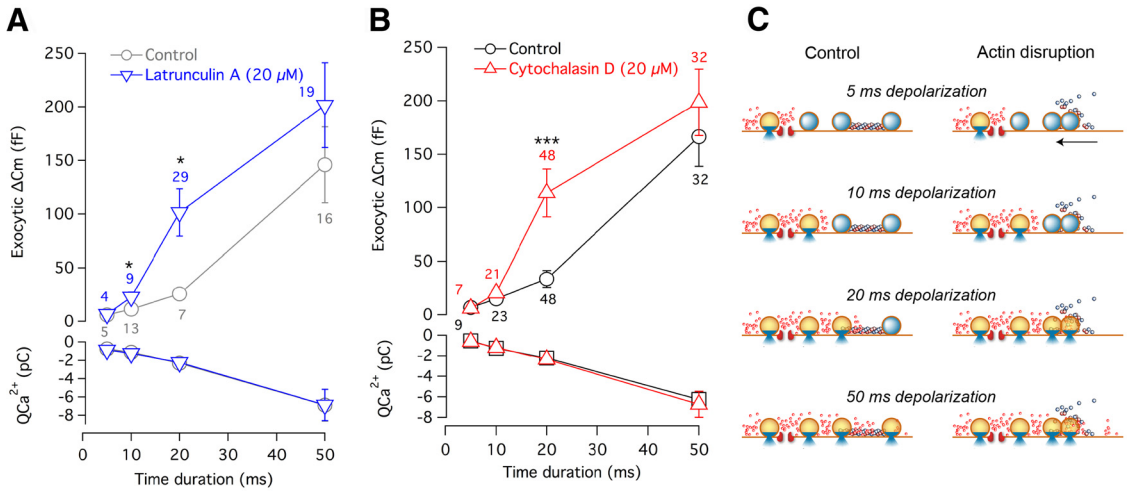

Figure 4. The facilitating effect of actin depolymerization is limited to 20 ms pulse duration. ***A***, ***B***, Exocytic ΔC_m (top) and the corresponding Ca^{2+} current integrals (QCa^{2+}, bottom) are represented versus the duration of depolarization for control IHCs (black) or infused with latrunculin A (LatA, 20 μM; ***A***) or cytochalasin D (CytD, 20 μM; ***B***). Calcium-triggered exocytosis was evoked in response to a single depolarization to −27 mV from a holding potential of −87 mV. Only one depolarization step was evoked per IHC. Numbers of IHCs recorded are indicated for each depolarization step. *$p < 0.05$; ***$p < 0.001$. ***C***, Hypothetical mechanism underlying the increase of exocytosis after actin filament depolymerization. In this scenario, actin filaments organize a fraction of synaptic vesicles with respect to the calcium channels. Disruption of actin leads to the displacement of a group of vesicles toward the calcium channels (arrow) so that these vesicles, which are normally recruited in response to a 50 ms pulse duration in the control condition, are mixed with the ones fusing within 20 ms. Alternative scenarios are possible (see Discussion).

trol: 6.8 ± 1.6 fF; latrunculin A: 6.6 ± 0.5 fF; cytochalasin D: 5.9 ± 1.1 fF) and longer (50 ms; control: 166.5 ± 27.7 fF; latrunculin A: 201.6 ± 39.5 fF; cytochalasin D: 198.5 ± 31.1 fF) pulses unchanged (Fig. 4). Because the exocytosis measurement assay used in our study is cumulative, the observed effect for a 20 ms pulse duration suggests that a fraction of vesicles normally recruited in response to a 50 ms pulse duration becomes available for release at a shorter pulse (20 ms) after the actin filament disruption.

Discussion

In our study, depolymerization of actin filaments increased IHC secretion. However, this effect was prevalent for an intermediate duration of stimuli (20 ms), excluding a facilitatory action on the RRP. We propose that the actin filament network might organize the location of a fraction of the SRP synaptic vesicles with respect to calcium channels.

Actin and hair cell exocytosis

In most of the studies, the role of the actin network has been inferred using drugs known to disrupt actin filaments (Casella et al., 1981; Spector et al., 1983; Cooper, 1987). Here, the comparable effects of latrunculin A and cytochalasin D support a similar mechanism of action: to inhibit actin polymerization. In addition, we validated the drug's specificity of action by coinfusing latrunculin A and phalloidin (an actin network stabilizer), a manipulation that prevented the action of latrunculin A and resulted in normal exocytosis. However, phalloidin itself does not alter the calcium-triggered exocytosis, suggesting that the actin network does not act in a dynamic fashion on secretion. Although actin filaments populate the hair cell cytoplasm (our study and Furness et al., 2005), synapsin is absent in hair cells (Layton et al., 2005; McLean et al., 2009; Uthaiah and Hudspeth, 2010). Synapsin is a synaptic protein known to interact with actin to cluster and mobilize a reserve pool of vesicles upon dephosphorylation and phosphorylation cycling, respectively (Greengard et al., 1993). Therefore, the lack of synapsin in hair cells calls for alternative mechanisms of action.

Potential mechanisms for the increase of exocytosis after actin disruption

The increase of exocytosis in response to a 20 ms pulse duration indicates that a higher number of vesicles undergo fusion during this depolarizing step. The increase in cell capacitance may therefore reflect the addition of a newly arrived vesicle pool, which is not recruited in normal conditions or the premature exocytosis of preexisting vesicles. In the scenario of additional vesicles being added to the preexisting pool, the amplitude of exocytosis after disruption should still be higher at 50 ms depolarization step because of the cumulative nature of our measurement of exocytosis. However, there is no difference between control and latrunculin A- or cytochalasin D-treated cells for longer depolarization pulses. This result can be explained if a subfraction of the SRP, which is normally recruited at 50 ms step depolarization, becomes available in advance and is engaged in release at a shorter step depolarization once the actin network has been disrupted. Different mechanisms may account for the change in stimulation–secretion coupling. For example, actin may set a position between the synaptic vesicles and the calcium channels to prevent a premature priming step of the vesicles (i.e., to become competent for fusion) by being closer to the calcium source (Voets, 2000; Neher and Sakaba, 2008; Lee et al., 2012, 2013). However, we cannot completely exclude that actin disruption converts reluctant synaptic vesicles into releasing vesicles within a spatially mixed population of vesicles (Sankaranarayanan et al., 2003).

In the hypothesis in which the SRP vesicles are remote from the calcium channels (Pangršič et al., 2015), actin filaments could slow down or prevent the movement of the synaptic vesicles toward the release sites, similar to chromaffin cells, in which the actin network forms a submembrane matrix that impedes morphological docking of granules to the plasma membrane (Vitale et al., 1995; Toonen et al., 2006). Together with an actin-free domain close to the synaptic ribbon, such a mechanism would allow the vesicles to quickly attach to the ribbon and avoid a traffic jam of vesicles for synaptic release. In the case of prolonged stimulation, calcium buildup at the synaptic region would favor

the depolymerization of actin because this process is calcium dependent (Bennett and Weeds, 1986), thus increasing the number of synaptic vesicles available for transmitter release.

If the resupply of the RRP by the SRP becomes faster after actin depolymerization, then the high rate of the RRP replenishment (Moser and Beutner, 2000; Spassova et al., 2004; Cho et al., 2011) may lead to SRP exhaustion (Voets et al., 1999), so longer pulses do not elicit an increase in exocytosis as we observed in our recordings. Therefore, the actin network might provide an efficient means for hair cells to operate vesicle replenishment at a high rate (Moser and Beutner, 2000; Spassova et al., 2004; Cho et al., 2011). In addition, our recordings at room temperature most probably underestimate the relevance of actin in vesicle trafficking because actin depolymerization, synaptic replenishment, and hair cell exocytosis are all temperature dependent (Wendel and Dancker, 1986; Kushmerick et al., 2006; Nouvian, 2007). Conversely, calcium channels themselves might undergo a higher mobility after the actin network disruption, as has been shown in cone and rod photoreceptors (Mercer et al., 2011). In this case, the extension of the area where calcium channels are confined could lead to the dispersion of calcium channels and enable the fusion of remote vesicles. According to this framework, the interaction between actin filaments and Cavβ2, an auxiliary subunit of the Cav1.3 channel expressed in the IHCs (Neef et al., 2009), has been reported recently (Stölting et al., 2015).

Finally, actin depolymerization may affect the spatial organization of organelles within the presynaptic terminal such as the endoplasmic reticulum (Joensuu et al., 2014). Bringing a local calcium source close to the active zone could amplify the calcium concentration through a calcium-induced calcium-release process leading to a larger secretion (Kennedy and Meech, 2002; Schnee et al., 2011; Castellano-Muñoz et al., 2015). Although we favor a change in the distance between synaptic vesicles and calcium channels because actin has been shown to have a structural role in other synapses (Cole et al., 2000; Cingolani and Goda, 2008), future examinations of the synaptic ultrastructure and calcium channel dynamics after actin disruption are required to test the above hypotheses.

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