Amplitude of Driven Oscillations of Bullfrog Sacculae Under Varied Conditions of the Otolithic Membrane

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ABSTRACT

It is well known that free standing hair bundles are capable of spontaneous oscillations within sacculus of the bullfrog, *Rana catesbeiana*. However, it has also been shown that these oscillations do not occur while the otolithic membrane is still attached to the hair cells. Under the same conditions, the hair bundles are able to typically move almost 4nm in amplitude. Different changes in the condition of the Otolithic membrane produced significantly greater displacement magnitudes. With the membrance removed from one side of the striola, hair bundles are capable of moving 13nm, demonstrating that in the prior condition, the movement is impeded by a net interference of the hair bundles. When the protein links connecting the hair bundles and otolithic membrane where removed, the stereocillia once again showed significant increase in movement with movement around 10nm. This showed that the weight of the otolithic membrane alone is not enough to stop the hair bundles from oscillating.

1. Introduction

In order to display such remarkable sensitivity hair cells use an active process to detect and amplify. The process behind this is non-linear which in bullfrogs and turtles has been observed to lead spontaneous oscillations *in vitro*. Although these spontaneous oscillations have exhibited amplitudes as great as 90nm [1], these oscillations have only been seen with free standing hair bundles. When these oscillations have been observed, the cells have had the otolithic membrane removed and are able to move independently of everything else. However, when the otolithic membrane has been left intact, the cells fail to show spontaneous oscillations.

It is unknown if the hair bundles have the ability to move the otolithic membrane even if they are given driven by an external power source. If the cells were exerting the maximum force they could possibly apply they may have the ability to move the membrane. However, it is possible that the cells may not be able to move the membrane even with the maximum force they are capable of exerting. One possibility is that the cells actually posses the ability to move while connected to the otolithic membrane, but are unable to because they are working against each other. Not all of the hair bundles point in the same direction and, since the otolithic membrane connects them all together, they will be fighting against one another to move. Furthermore, the cells will not spontaneously oscillate with the same frequencies or phase [2], which would result in minimal net movement. Even if the cells were able to operate with the same phase and frequency, the net movement would likely be negligible because they point in opposite directions. As figure 1 illustrates, the hair cells

either point inward or outward depending on which side of the striola they are on. As a result there is a net cancelation of the direction of movement. However, there is generally a greater number of cells on one side of the striola than the other so if there was sufficient force exerted the cells should be able to move the otolithic membrane. To test this theory, we peeled off most of the membrane on one side of the striola and left the system in a state where most of the cells will be working together. We then stimulated the cells electrically so they would all be driven with the same phase.

The other major rationale for why hair bundles don't naturally move under the otolithic membrane is due to the composition of the filaments connecting them to the membrane If this connection is too rigid, then the cells would not be able to oscillate without the entire otolithic membrane moving too. As a result, if the links were removed with protease then the cells may be able to move under the covering membrane.

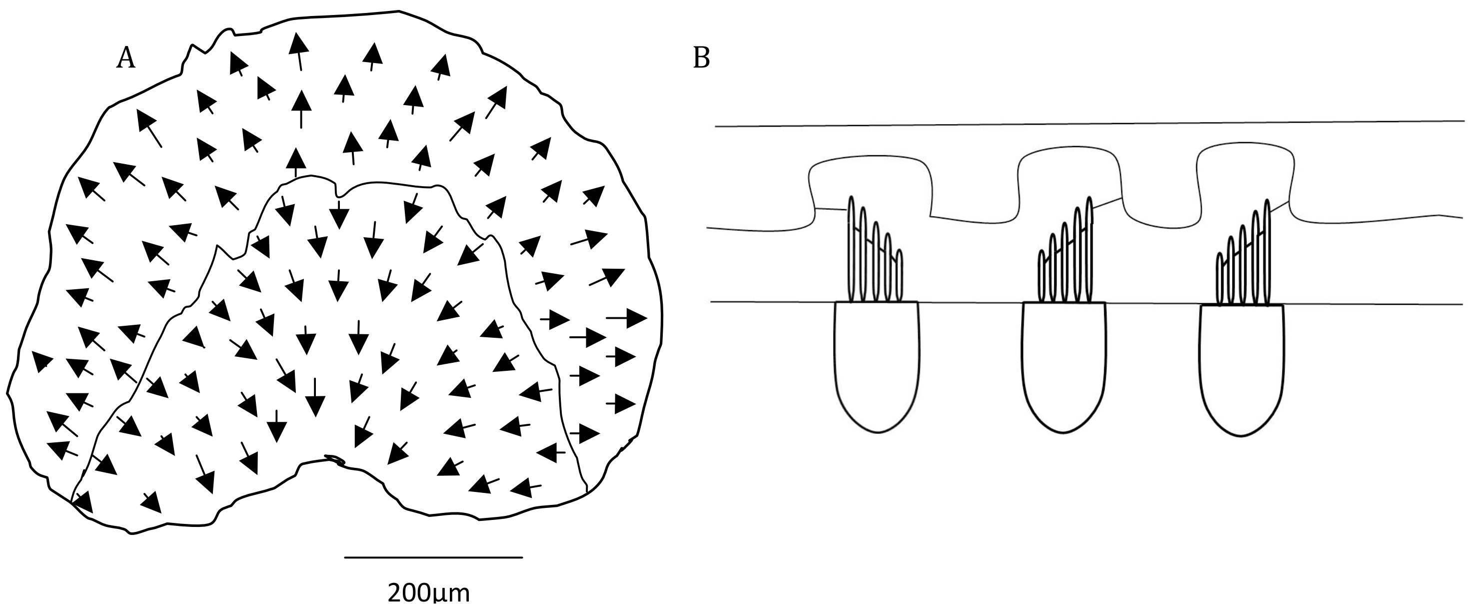

Figure 1: A) The distribution of positive displacement for hair bundles a bullfrog macula. The arrow indicates the direction of the hair bundle's positive movement. The line through the center represents the striola. B) A diagram of hair cells, the otolithic membrane and the connection between them.

2. Materials and Methods

Experimental Preparation. Sacculi of adult bullfrogs (*Rana catebeiana)* were excised from the inner ear. The macula was removed and transferred to a two-compartment

chamber. The apical and basolateral surfaces of the epithelium were immersed in oxygenated saline solutions to simulate natural circumstances. Artificial endolymph containing 117.5 mM K+, 2 mM Na^+, 0.25 mM Ca^{2+}, 118 mM Cl^-, 3 mM D-glucose, and 5 mM HEPES at pH 7.3 was placed on the apical surface. The bottom compartment was filled with artificial perilymph containing 110 mM K^+, 2mM Na^+, 1.5 mM Ca^{2+}, 118 mM Cl^-, 3 mM D-glucose, 1 mM sodium pyruvate, 1 mM creatine, and 5 mM HEPES at pH 7.3.

Stimulation. The cells of the macula were forced to oscillate by fixing the current traversing the cell membrane. This was done by using a current clamp to send a sinusoidal signal to the sample. The current was set to an amplitude of 50μA and a frequency of 10Hz.

Measurement. The movement of the cells was imaged with an Olympus BX51WI microscope with white-light transmission illumination provided by X-Cite 120 metal halogenide lamp. A 20x water-immersion objective with N.A. of 0.95 was used, with further magnification provided by a double-gauss variable-focus lens, for a total magnification of ≈ 385x. To minimize mechanical noise, the microscope was mounted on a vibration-isolation table inside an acoustically-isolated chamber (Industrial Acoustic Company). All images of hair bundle oscillations were recorded with a CMOS camera (Photron FASTCAM SA1.1) containing 1024x1024 pixels, each 20 μm in size and with 12-bit depth. The recordings were triggered by LABVIEW. The spatial scale of the image projected onto the camera chip was calibrated by viewing a 600 line pair per millimeter Ronchi ruling, yielding 60 nanometers per pixel.

Membrane Peeling. In order to remove the membrane from one side of the striola, an eyelash tool was used to peel back the otolithic membrane from the unwanted side. This left the membrane still attached to some of the hair cells on the undesired side of the membrane, but the net change in the overall ratio of cells on either side of the striola was great enough to neglect this.

Protein Removal. The perilymph was removed and the Maculae were exposed for 35 minutes to 67 $\mu g \cdot ml^{-1}$ protease at room temperature. The protease was washed away and the perilymph was replaced. As a positive control, after imaging the cells movements with these conditions the otolithic membrane was removed and the macula was once again stimulated.

Cell Comparison. In all cases, we first measured the movement of the cells with the otolithic membrane completely intact and no external stimulation applied. The macula was then stimulated and the movement of the hair bundles was observed. Afterward, the desired change in the condition of the otolithic membrane was implemented and the same area of the macula was once again observed while it was being driven. Finally, when applicable, the entire otolithic membrane was removed and the process was repeated once more. Since the same location on the macula was observed when the same preparation was used to analyze different conditions, we were able to identify specific cells that were present in all cases.

3. Analysis

Motion tracking. To extract the motion of a hair bundle as a function of time, used a MatLab program to determine the position of the tallest row of stereocilia in each frame of the video record and then track it's movement (2). Hair bundle movements were tracked only in the x-direction. Consequently, due to an unavoidable distribution in the orientation of the cells, this method produced an underestimate of the amplitudes of oscillation. This was not a major worry for this study because this effect was fairly small and we were mostly concerned with the relative movement of the hair bundles under the different conditions we imposed. The motion of the hair bundles was then analyzed using Mathematica to fit the motion of the stereocillia to a 10Hz sine wave to find the magnitude of oscillation at the driving frequency. If the hair bundles movement did not appear to match a 10Hz frequency the data from those bundles was ignored. This was caused by either too much noise obscuring the signal or in some cases, the cells had died and had cease to respond to electrical stimuli.

Imaging the sacculus. The tiled images were created using two-dimensional tracking software (1). This process allowed us to match up cells from different images. From this we were able to compare the oscillations of the same cells under identical circumstances to find the amount of variety that is inherent in our methods of measurement.

4. Results

The size of the oscillations observed increased significantly in both the case of peeling off the membrane from one half of the striola and when protease was used to dissolve the protein links to the otolithic membrane. Table 1 shows the hair bundle response under the different conditions. Although there is an amplitude of over one nanometer for the unperturbed without stimulation, this was likely not movement caused by oscillations of the hair bundles. The displacement plots of these hair bundles suggest that this movement can be attributed to noise. Likewise, some of the unperturbed cells that were being stimulated showed no movement above the noise level. However some of the cells did show movement as great as 8nm, whereas the maximum movement of the cells without stimulation was just over 3nm.

In all cases when the same cell was analyzed for different conditions, the hair bundle exhibited larger amplitude of oscillation. Figure 4 shows typical results. In the case where half the membrane was peeled away, the cells which remained under the membrane showed a two-fold increase in their magnitude of oscillation. In the most extreme cases, the oscillation size was more than tripled. When the protein linking the hair bundles to the otolithic membrane were digested, the oscillations approximately tripled in magnitude in each case where the same cell was tracked under all conditions. When these same cells had the otolithic membrane completely removed there was another increase in magnitude with a range from 10% to 100%.

Condition of Macula	Mean Oscillation (nm)	Standard Deviation (nm)	Range (nm)	N
Unperturbed, no stimulation	2.19	0.92	0.79-3.14	5
Unperturbed	3.82	2.23	0.19-8.07	16
Membrane Peeled	12.85	4.67	5.80-22.46	20
Protein Links Removed	10.29	3.40	4.91-17.74	18
Membrane Removed	14.32	7.81	6.11-35.63	18

Table 1: Displacement response for hair bundles under different conditions. In all conditions, unless specified otherwise, the hair cells were being stimulated electrically. The range gives the minimum and maximum displacements seen after removing outliers. N refers to the number of cells sampled with the specified condition.

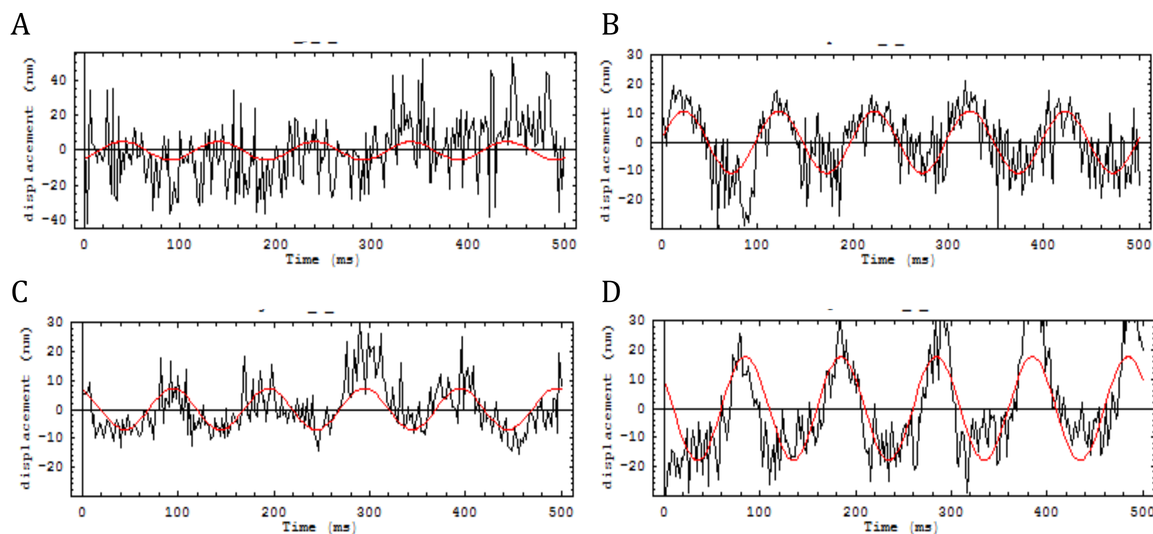

Figure 2: Representative results for different conditions of the otolithic membrane. A) No alteration to the otolithic membrane. B) Membrane peeled to the stiola. C)Protein links digested D) Otolithic membrane removed.

5. Discussion

Previous studies have shown that the hair cells of bullfrog sacculus lack sufficient power to oscillate spontaneously while attached to the otolithic membrane. The purpose of this study was to determine if the hair bundles could move the otolithic membrane if they were given sufficient external stimuli. In addition, the cause for the hair bundles' inability to oscillate spontaneously under the otolithic membrane is unknown. This phenomenon is most likely the result of one or both of two effects. First, the cells of the macula do not share a common direction for their positive displacement. Therefore, it is useful to study the macula with conditions altered to allow for there to be a greater degree of cooperation among the hair bundles. The other possible explanation for this is that the protein attaching the hair bundles to the otolithic membrane is too rigid to allow movement. This can be examined by digesting the proteins.

The preliminary results suggest that even when supplied with enough energy to saturate the hair bundles' movement, they may not be able to move the otolithic membrane. There was an increase in the mean amplitude of displacement, but it was not enough to conclude that there was significant improvement. With electrical stimulation the hair bundles did increase their movement by nearly 4nm, but the noise level was around 2nm. It would seem that there is a slight increase in movement of the hair bundles, but not enough to definitively say that this is due to the external stimulation.

It is clear that peeling off part of the otolithic membrane gave a significant increase in the hair bundles' movement. When the otolithic membrane was removed from one side of the striola, oscillation size was increased, on average, by a factor of six. Furthermore, every hair bundle tracked moved with a displacement greater than the mean of the hair bundles before the membrane was peeled back.

Similar results were seen when the protein links to the otolithic membrane were removed. Again, the minimum oscillations were greater than that of the mean prior to removing the connection. The oscillation amplitudes under these conditions were not as large as they were for disconnecting half the membrane. It is possible that the source of the increase in magnitude could be a result of them no longer being connected via the otolithic membrane. With the protein links gone, the cells are free to move far more independently of the otolithic membrane. The amplitude of oscillation was still less than that of the other case. This could mean that the cells are either still connected to each other as a result of the mass of the otolithic membrane pushing down on them or they simply can't move the membrane as much regardless of how they are connected. However, it isn't even necessarily the case that removing the protein links has a lesser effect. The two conditions were observed using different maculae. Therefore, further data will be needed to determine if there is significant difference between the two conditions.

Neither condition where the otolithic membrane was in some way still on the macula showed as great a magnitude as that of the hair bundles with the entire membrane removed. Although the mean oscillations of under these condtions were only 2nm greater in magnitude than that of the hair bundles with the otolithic membrane peeled back, it still seemed that the oscillations were overall larger without the membrane at all. The large uncertainty in the amplitude size of the hair bundles with the otolithic membrane removed reflected the large range in the magnitudes seen. There were four hair bundles with movement exceeding 20nm with the largest at 35nm. There were also many hair bundles with oscillations well below the mean. In addition, there were several cells that were clearly dead after excising the membrane. Therefore it is safe to conclude that if all the cells had been healthy, the mean oscillations would probably have been greater. This is something that could be easily determined by comparing the transduction current of the cells before and after digestion to quantify the health of the cells. Also, part of the cause of deterioration of the cells may have stemmed from the cells having already been exposed to large electrical stimuli far beyond their natural range and these cells were examined after having been *in vitro* for approximately 45 minutes longer than the ones that just had the membrane peeled from half the sample. To determine if either of these did play a role it would be advisable to examine hair bundles with the membrane completely removed without using them for anything else beforehand.

6. Acknowledgements

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7. References

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