Possible Mechanism Behind Calcium Dependence in the Adaptation Process of Mammalian Cochlear Hair Cells

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Accepted for publication: 2023
Published Date: October 2023

Abstract

Hair cell adaptation is an important process that influences our auditory sense. However, this process still has many unexplained aspects, such as the mechanism behind it (as reviewed in Farhadi et al., 2021). Previous research indicates that this mechanism is related to calcium input and the phospholipid PIP2 (as reviewed in Eatock, 2000; Farhadi et al., 2021). Calcium input is required for the mechanoelectrical transduction channel (MET) to function, originating the adaptation process (as reviewed in Eatock, 2000); PIP2 may influence the proteins TMIE and TMC1 and the conductance of the MET channels (Cunningham et al., 2020; Farhadi et al., 2021). Therefore, I propose that in order for calcium to flow across the MET channels, PIP2 binds with TMIE, triggering TMIE to bind with TMC1 which alters the shape of the MET channels and thus affects channel conductance. My research found that the adaptation process is indeed calcium-dependent and that PIP2, TMIE, and TMC1 bindings very likely affected MET channel conductance. These findings may resolve the mystery around the mechanism of hair cell adaptation and bring more insight into the auditory function including potentially detrimental malfunctioning such as deafness. My findings suggest there is a chain behind the mechanism and prove my hypothesis to be correct. I hope my findings can contribute to the scientific community as a foundation for future research on the mechanisms of adaptation.

Keywords: Hair Cells, TMIE, PIP2, TMC1

1. Introduction

For several decades, the adaptation process of hair cells has been researched. The adaptation process has many functions including amplifying the sound received by hair cells, regulating the frequency of sounds, and setting the hair cell's resting potential (Ricci et al., 2005; Farris et al., 2006; Johnson et al., 2011). It is evident that adaptation is an important aspect of hair cells to protect hearing ability in organisms. Based on previous research, a general model of adaptation was proposed and is still widely recognized today. In this model, the adaptation process requires calcium entry into stereocilia (as reviewed in Eatock, 2000). However, recent papers argue that in mammalian hair cells, the adaptation process may be calcium-independent (Peng et al., 2013). It is also proposed that PIP2, a kind of phospholipid, and the mechanoelectrical transduction (MET) complex proteins TMIE and TMC1 are involved in the functioning of MET channels, which are important for calcium flow and thus the adaptation process. However, despite the current knowledge, the underlying mechanisms for adaptation are still not fully known (as reviewed in Farhadi et al., 2021). In this research paper, I aim to examine whether the

adaptation process is calcium-dependent, and further investigate the role of PIP2, TMIE, and TMC1 in adaptation.

Hair cell adaptation is closely related to MET channels. The hair cell's apical surface projects a specialized hair bundle composed of stereocilia, with each stereocilium made of actin filaments (as reviewed in Hudspeth, 2005). The stereocilia form in three rows of different heights with tip links attached to connect neighboring pairs of stereocilia in adjacent rows (Pickles et al., 1984). On the tip of the stereocilium is found mechanoelectrical transduction (MET) channels. When mechanical stimuli reach the hair cells deflection occurs in stereocilia due to the sound wave, and the MET channels open. The MET current depolarizes the hair cell, which leads to neurotransmitter release at the basolateral end of the cell.

For a while, PIP2 has been a central subject in the research for the mechanism behind adaptation. In 2004, research was done to assess the influence and role of PIP2 on adaptation (Hirono et al., 2004a). The experiment found that depletion of PIP2 lowered the rate of hair cell adaptation in frogs. A more recent paper by Cunningham et al. found that PIP2 affects MET channel conductance and ion selectivity at least in part via the protein TMIE (Cunningham et al., 2020). Meanwhile, research also points out that MET channel proteins TMC1/2 along with PIP2 and TMIE may be functioning together to make the MET channels function properly (as reviewed in Farhadi et al., 2021). However, there are only simulated models that speculate on how the three components work together.

TMIE protein is also important for adaptation as it may also affect the conductance of MET channels and thus the currents during adaptation. A study found that TMIE protein binds with lipids via a structure called C-terminal, and one of the binding sites overlaps with the domain important for binding with TMC1, suggesting that TMIE and lipid binding will also affect TMIE and TMC1 binding; the same study also suggests that mutations in TMIE next to the binding sites for TMC1 decrease MET channel currents when hair cells are stimulated (Cunningham et al., 2020). However, TMIE does not seem to be a pore-forming subunit of the MET channels (Zhao et al., 2014).

A study of TMC 1 protein structure suggests that TMIE triggered by force would bind with TMC1 (Jeong et al., 2022). Since TMC1 has pores that likely regulate ion permeation of MET channels, the combination would allow for ion permeation. Experiments done by Cunningham et al. also seem to confirm this proposed mechanism. Based on the evidence above, PIP2 binding may affect TMIE, which in turn affects TMIE binding with TMC1. TMC1 may be a pore-forming subunit of the MET channels. If only TMIE binding with TMC1 would result in the correct shape for TMC1 to allow calcium flow, this could explain the effect of TMIE on channel conductance, therefore influencing hair cell adaptation as the current required for adaptation depends on calcium flow.

To test my hypothesis, I used mouse cochlear hair cells combined with calcium imaging and mutations of TMIE to assess the role of calcium and PIP2 in the adaptation process. My results show that mammalian hair cells seem to be calcium-dependent despite previous research and that a lack of PIP2 results in slower adaptation and abnormal calcium movement across the MET channels. Then I tested PIP2, TMIE and TMC1 binding using colP intensity to see if unsuccessful binding between PIP2 and TMIE would result in unsuccessful binding between TMIE and TMC1. This seems to be the case; to ensure that this binding process leads to a decrease in calcium conductance, I measured single-channel currents when there's PIP2 depletion. So far, the experimental evidence seems to prove that my hypothesis is correct.

2. Methods and Materials

Preparation
This technique is adapted from Beurg et al., 2009.

Electrophysiology
This technique is adapted from Beurg et al., 2009.

Two-Photon Imaging
This technique is adapted from Bender and Trussell et al., 2009.

Calcium Imaging
This technique is adapted from Bender and Trussell et al., 2009. A two-photon imaging technique was used

for more precise and detailed imaging. Fluorescence was used to distinguish Ca2+ ions. Alexa 594 ($20\mu m$, red) was used as the control dye. The laser used in the two-photon imaging equipment was tuned to 810 nm (Bender and Trussell, 2009). To visualize the calcium movement, I used GCaMP6, a genetically encoded calcium indicator.

Pharmacology
This technique is adapted from Caprara et al., 2020.

Mutation in Mice
This technique is adapted from Tsai et al., 2012, Zhao et al., 2014, and Cunningham et al., 2020.

Single MET Channels
This technique is adapted from Ricci et al., 2003.

Protein Extraction and Purification
This technique is adapted from Jeong et al., 2022.

Western Blotting and Immunology
This technique is adapted from Cunningham et al., 2020.

Graphing
The graphing software Prism (Graphpad.com) was used to generate graphs and some statistics. Some of the graphs of current were drawn using drawing software such as Freeform. The remaining graphs came directly from other research papers, with some modification as needed.

3. Results

The mechanism behind the adaptation process is of main interest to the research. There has been little research done on this area and previous papers indicate that PIP2, TMIE, and TMC1 are involved in this process. My research aims to prove that the binding of PIP2 and TMIE affects the binding of TMIE with TMC1, which in turn affects MET channel conductance as TMC1 may be a pore-forming subunit for the channels, regulating calcium flow by shape.

The adaptation process is calcium-dependent
First, I measured the current during changes in voltage between positive and negative values (Figure 1B). I maintained the cell voltage at -100 mV. When the membrane voltage was increased to +80 mV, the outward current through voltage-gated channels increased as well. However, these currents did not include MET channels. When I then applied mechanical stimuli halfway through +80 mV, the MET channels were activated and an outward, non-adapting current was observed. As positive voltage makes calcium flow outward rather than inward of the hair cells, the lack of adaptation during positive voltage shows that calcium entry is necessary for the adaptation process. Then I lowered the voltage again to -100 mV to create an inward driving force on K+, and most important for this study, Ca2+. With an inward driving force on calcium now present, I observed a clear adaptation process in the inward MET currents. This suggests that calcium does influence the adaptation process. Based on this hypothesis, I reasoned that calcium imaging during this inward current will confirm the link between the -100 mV membrane potential and the influx of calcium through the MET channel (Beurg et al., 2009).

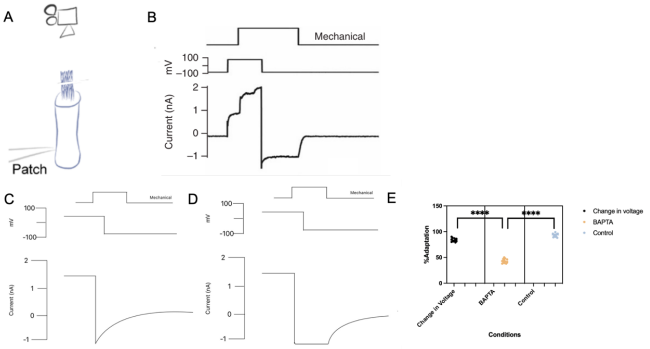

Figure 1. Adaptation process under the absence and presence of calcium. (a) A schematic representation of the experimental setup to measure the mechanoelectrical (MET) current of hair cells. The drawing features a mammalian hair cell, with the patch indicating the electrode recording the currents of the channels. The structure above the hair cell is the water jet which provides the mechanical stimulation (Drawing adapted from Figure 4 of Caprara et al., 2020). **(b)** Voltage clamp measurements of Ca2+ current of hair cells initially held at a voltage of -100 mV. When the voltage was changed to +80 mV, then the mechanical stimulus (top trace) opened MET channels but no calcium entered the cell. When the Vm was moved back to -100 mV, the negative inward current is seen. The negative current shuts off when the mechanical stimulus is stopped. Data were obtained as an average of 12 mice. The current is expressed in nA and the voltage is expressed in mV (Drawing adapted from Figure 1 of Beurg et al., 2009). **(c)** High magnification image of the current in hair cells when the membrane voltage was changed from +80 mV to -100 mV. Mechanical stimuli were applied during +80mV until sometime into the period when the voltage became -100mV. **(d)** Under similar conditions as (c), BAPTA was used. Note the delay in the adaptation process. **(e)** Graph showing the percent adaptation observed in WT (panel B; black symbols), control (panel C; blue symbols), and BAPTA (panel D; yellow symbols). The difference

between them is significant.

To test my hypothesis that the influx of calcium is what caused the adaptation of the negative, inward currents, I artificially reduced internal calcium concentration. I did this by using BAPTA, a calcium chelator that decreases the number of free calcium ions by binding with them. Using 5 mM BAPTA to reduce internal calcium concentration, I observed a significant delay in the adaptation of inward Ca2+ currents (Figure 1C, D). To quantify the effect on adaptation, I measure the percent of adaptation of hair cells with and without BAPTA application (Figure 1D, E). Overall, cells with BAPTA had lower percent of adaptation, confirming my hypothesis that the adaptation process was influenced by a lack of calcium.

Together, these two experiments show that hair cells lacked adaptation of MET currents when calcium entry is blocked by outward driving force or internal calcium buffers. Thus, from the above experiments, I concluded that overall, the adaptation process in mammalian cochlear hair cells is calcium-dependent.

PIP2 affects the rate of adaptation

After investigating the role of calcium, I hypothesized that PIP2 would affect calcium current as it was demonstrated by previous research that PIP2 affected MET channel conductance (Cunningham et al., 2020). Thus, I tested the effect PIP2 had on the adaptation process. Previous research has demonstrated that in frog saccular hair cells, the chemical blockage of PIP2 results in a decrease in adaptation rate (Hirono et al., 2004b). In this research paper, I utilized cochlear hair cells in genetically modified mice that had PIP2 depletion. As far as I am aware, no previous research paper examines the actual calcium movement when PIP2 is depleted. Given the speculation on whether the lack of PIP2 and TMIE binding resulted in abnormalities in the conductance of MET channels (as reviewed in Farhadi et al., 2021), I set out to test this hypothesis and further investigate the possible mechanisms of PIP2 and other proteins.

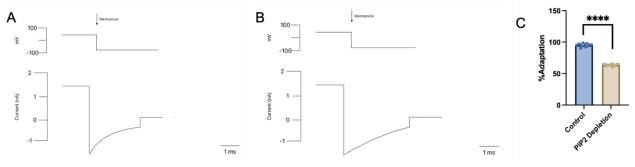

Figure 2. Depletion of PIP2 leads to slower adaptation. (a) The average current of hair cells when mechanical stimuli and a change in voltage from +80 mV to -100 mV were provided in the control group where PIP2 was not depleted. **(b)** The average current of hair cells with mechanical stimuli and a change in voltage from +80 mV to -100 mV were provided in the experimental group where PIP2 was depleted. **(c)** The percent adaptation of the control (a) and the PIP2 depletion (b) groups.

I investigated if PIP2 depletion would affect the adaptation process. Using molecular biology techniques, the p.R82C gene, which encodes for PIP2 in mice of the experimental group was removed resulting in a PIP2 depletion. I then separated the mice into mutant (PIP2 depletion) and control groups in order to test my hypothesis that hair cells with PIP2 depletion will not be able to properly form calcium currents. I then performed an experiment where hair cells were held at +80 mV and then suddenly decreased membrane potential to -100 mV. I measured the current of the hair cells in both mutant and control groups and found that it was much more difficult for the mutant group to perform the adaptation process (Figure 2A, B). Data analysis confirmed this as the percent adaptation in the mutant group was significantly lower than the control (Figure 2C). This result confirms the findings of Hirono et al. and shows that PIP2 is important for the adaptation process (2004). I next examined a potential mechanism for PIP2 in the adaptation process.

PIP2 affects calcium movements across MET channels

While my findings show that PIP2 affected the adaptation process, its mechanism of action was not clear. Past research suggests that PIP2 affects the MET channel conductance of calcium (as reviewed in Farhadi et al., 2021). However, this prediction has not yet been tested. I hypothesized that the PIP2 effect on calcium movement across MET channels could provide a mechanism for the calcium dependence of adaptation of MET currents in hair cells.

I imagined that without PIP2, calcium entry would be abnormally slow (Figure 3A). Using calcium imaging with green fluorescent (Fluo 4 fluorescent) and using red fluorescent as the control (Alexa 594), I measured the calcium at the MET channels over time. I used % Δ G/R to show the relative amount of calcium, with ΔG being the amount of calcium moved across the channels and R the red control. More % ΔG/R meant that there was more calcium entry. The results showed that in a given amount of time 200 ms more calcium flowed across the channels in the control group than

the group with PIP2 depletion (Figure 3B). To be sure, I conducted a data analysis and found that the group with PIP2 depletion did have significantly less percent of ΔG/R (Figure 3C).

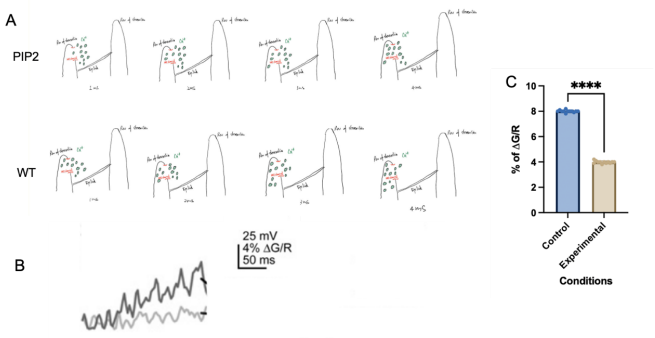

Figure 3. Depletion of PIP2 leads to slower calcium movements across MET channels. (a) A schematic drawing of the movement of calcium ions across MET channels according to our hypothesis. The upper part is the control group while the lower part is the experimental group. The picture shows, in an idealized fashion, how calcium moves in 4 mS. **(b)** When the time spent is the same, the graph records the maximum % ΔG/R reached in a certain amount of time by the control (upper, black) and the experimental (lower, gray) groups (Drawing adapted from Figure 5 of Bender and Trussell, 2009). **(c)** Taking the average maximal value from (b) to draw a column graph. The x-axis is the control and experimental groups while the y-axis is the percent of ΔG/R. The two groups were significantly different.

The results proved that PIP2 impacted the flow of calcium across the MET channels. This may also further prove that PIP2 directly or indirectly regulates the calcium conductance of the channels, therefore influencing the adaptation process mentioned in the last section. Now I wanted to see exactly how PIP2 brought about this effect.

The binding of PIP2 and TMIE leads to the binding of TMIE and TMC1

PIP2 may indirectly affect calcium conductance by TMIE and TMC1 bindings. Previous research suggests that TMIE has binding sites for PIP2 that may affect its binding with TMC proteins and calcium current (Cunningham et al., 2020). There was also research on TMC1 which suggests that TMC1 acts as a pore-forming subunit of the MET channels and may regulate calcium conductance (Jeong et al., 2022). Based on these papers, I next hypothesized that PIP2 and TMIE binding would result in TMIE binding with TMC1. In order to investigate this mechanism, I hypothesized that disruption to the binding of PIP2 and TMIE will lead to loss of binding of TMIE and TMC1, thus affecting calcium conductance. I measured colP intensity as an indicator of the binding of TMIE and TMC1 (Cunningham et al., 2020). To disrupt the binding of PIP2 and TMIE, I used the mutated mice in which the parts on the C terminal of TMIE binding to PIP2 were changed, making it impossible for PIP2 to bind so that PIP2 and TMIE combinations would be unsuccessful. I also investigated the binding between TMIE and TMC1 proteins from mice cochlear hair cells.

The binding between TMC1 and TMIE after PIP2 binds unsuccessfully to TMIE would be weakened according to my theory. I chose twelve mice for the control and the group with unsuccessful PIP2 binding respectively. Then I ran the molecules through western blotting, using co-immunoprecipitation to show if TMC1 bound to TMIE. The amount of binding was marked as colP intensity: the higher the intensity, the higher the chances for binding. I found that the control group had significantly more successful bindings than the mutated group (Figure 4A). Next, I investigated if the inability of TMIE to bind with TMC1 would affect the calcium conductance. I performed mutation on the TMIE protein to disable its binding sites for TMC1. When provided with several different levels of mechanical stimuli, the control group had corresponding current while the group with TMIE mutation had almost no current (Figure 4B). I plotted the graph of MET channel currents against different degrees of mechanical stimuli and confirmed this result (Figure 4B).

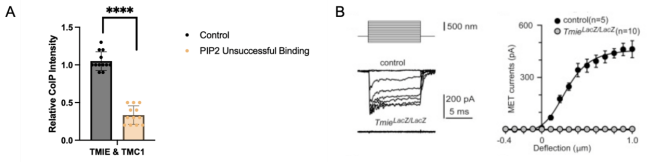

Figure 4. Unsuccessful binding of PIP2 with TMIE leads to unsuccessful binding between TMIE and TMC1, which in turn affects calcium currents. (a) The column graph of the relative ColP intensity of TMC1 and TMIE binding. The black bar represents the control condition while the yellow bar represents the condition of unsuccessful binding of PIP2 and TMIE. The ColP intensity under the two conditions is significantly different. **(b)** Hair cells received different mechanical stimuli (upper left). The control hair cells showed different average currents while the TMIE mutated hair cells showed no change in currents (lower left). The right side of the figure shows a graph of the two conditions. (Drawing adapted from Zhao et al., 2014)

These results show that the binding of PIP2 to TMIE was crucial for TMIE to bind with TMC1, and that not only PIP2, but also TMIE and TMC1 affected calcium conductance. This proved my hypothesis on the

binding of PIP2, TMIE and TMC1. However, further proof of the molecules' effects on single-channel currents was needed.

PIP2 and TMIE affects calcium current in single MET channels

To test if PIP2 and TMIE also had an influence on single-channel conductance I made the following investigation. So far, my experiment has been focused on average MET channel current, and a more precise measurement for proof may be needed. Therefore, I used PIP2 and TMIE mutation to measure the current changes and tried to manipulate the concentration of PIP2.

I needed to prove that PIP2 and TMIE affected single-channel currents. Thus, I used genetic mutation to knock out TMIE and deplete PIP2. I compared the current change of mice in different conditions when mechanical stimuli were provided. The results showed little change in calcium current when PIP2 or TMIE was removed compared to the control group (Figure 5A). This showed that both PIP2 and TMIE were required for calcium current in single channels.

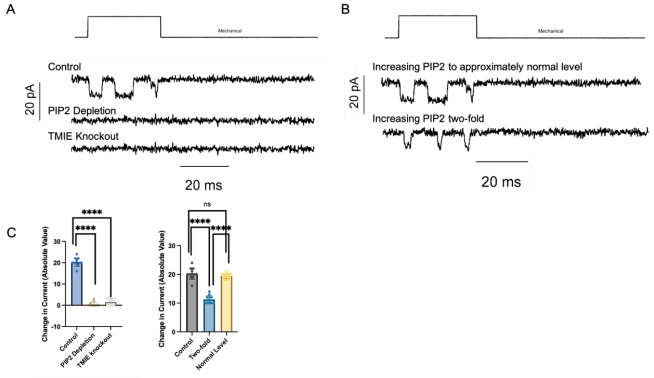

Figure 5. Single MET channel current is affected by PIP2 and TMIE. (a) The current of the MET channels was recorded during mechanical stimuli. The upper current is the control group, the middle is the group with PIP2 depletion and the lower current is the group with TMIE knockout. (Drawing adapted from Zhao et al., 2014) **(b)** PIP2 amount was increased in hair cells with PIP2 depletion. The lower current is the average when the PIP2 level was increased to two-fold of the original amount. The upper current is when the PIP2 level is fully restored. (Drawing adapted from Zhao et al., 2014)

Since the absence of PIP2 reduces calcium current, increasing the amount of PIP2 may restore calcium entry. I raised the PIP2 level in hair cells with PIP2 depletion. When the PIP2 amount was increased to two times the original amount the current change became much greater. When the PIP2 level was increased to the normal level the change in current was similar to that of the control group (Figure 5B). I performed a statistical analysis and found that in the situations when PIP2 was depleted or TMIE knocked out, the change in current was significantly smaller than in the control group (Figure 5C). Furthermore, when I raised the level of PIP2 to a normal amount there was no significant difference between the control and the subject in question (Figure 5C). It is evident that restoring the amount of PIP2 increases the MET channel conductance.

Thus, PIP2 influenced MET channel conductance just as TMIE did. Combining this information with the previous research on the link between PIP2, TMIE, and TMC1 binding provided evidence that the binding of PIP2 and TMIE as well as the binding of TMIE with TMC1 affected the MET channel conductance.

4. Conclusion

This research paper examined the roles of calcium and PIP2 in the adaptation process of mammalian cochlear hair cells. By changing the driving force on calcium ions, I was able to create calcium depletion and the graph of the current showed that the adaptation process is calcium-dependent. The mutation that led to a depletion of PIP2 created a faster decline in current, slower adaptation rate, and faster calcium movement compared with the control group, thus proving that PIP2 is essential for the adaptation process. Based on the abnormal calcium movement and previous research, I hypothesized that PIP2 was indirectly responsible for calcium conductance in MET channels. My findings support this hypothesis and show that the binding of PIP2 and TMIE is required for binding of TMIE with TMC1, which in turn affects the calcium conductance and subsequently the adaption of MET currents.

I found that the adaptation process is calcium-dependent which is supported by most research but also contradicted by a few including a 2013 paper (Peng et al., 2013). The effect PIP2 had on the adaptation rate is also confirmed by a 2004 paper (Hirono et al., 2004b). However, no research until now has looked at the calcium movement under PIP2

depletion, nor has any research linked the bindings of TMIE, PIP2, and TMC1 together, though there has been speculation on PIP2 affecting the conductance of MET channels (as reviewed in (Farhadi et al., 2021).

There are aspects of the research that are imperfect and may yield errors. The binding of TMC1 and TMIE could only be assessed through the intensity of colP, which leads to certain ambiguity as to the reality of TMC1 and TMIE binding. More importantly, I only demonstrated that PIP2 and TMIE had an unsuccessful binding that likely led to an unsuccessful binding between TMIE and TMC1, but I was not able to eliminate all possible interfering factors. I was also not able to show that it was a complete cause-and-effect relationship. This causes problems that the unsuccessful binding between TMIE and TMC1 may arise from some other steps that were triggered by the unsuccessful binding between PIP2 and TMIE, thus missing certain steps in this chain of mechanism. Future research could continue to perfect the mechanism of PIP2's function in the adaptation process, as the research did not fully construct a causational relationship. In this research paper, I only investigated PIP2's effect on calcium current. However, as my theory was that PIP2 binding affects TMC1 binding and that TMC1's pore affects ion conductance, it would be possible for PIP2 to affect ion conductance overall.

Future research could investigate if PIP2 would also affect ion conductance and if it is only limited to mammalian cochlear hair cells. Researchers could also look at the molecular level changes of PIP2, TMIE, and TMC1, especially when they bind. It may be these conformational changes that affect the MET channel conductance. My future research would focus on the exact way these molecules change the MET channels' conductance—I hypothesized that TMC1 was part of the subunit of the MET channel, changing the channel's shape to regulate calcium entry.

Hair cells probably developed the adaptation process to protect the auditory function. Adaptation allows hair cells to regain their normal calcium current and lowers the stimulation from sounds. It is also reasonable for cells to develop such a complex mechanism for ion regulation, as the voltage and functions of the cell depend entirely upon ion flow. Having regulations for calcium and other ions is a safeguard against unwanted ions and thus malfunctioning.

The findings of the study are important as they may open a new field of research in which the interactions between PIP2, TMIE, and TMC1 are studied further. It may also be able to help solve the long-standing puzzle of the inner molecular mechanisms of hair cells. By learning more about the adaptation process, this paper is helping scientists to know more about some of the crucial functions of hair cells. The adaptation process has many functions including amplifying the sound received by hair cells, regulating the frequency of sounds, and setting the hair cell's resting potential (Ricci et al., 2005; Farris et al., 2006; Johnson et al., 2011). Investigations on adaptation may also be important for further understanding of deafness.

In conclusion, the mechanism behind the calcium flow of adaptation has been suggested. I found that the adaptation process in mammalian cochlear hair cells is calcium-dependent and that PIP2 likely regulates the calcium conductance of MET channels. Based on my experimental findings, I suggest that PIP2 and TMIE binding regulates TMIE and TMC1 binding, which alters the shape of TMC1. TMC1 being a pore-forming subunit of the MET channels likely influences the channels' shape, thus regulating the calcium conductance of the channels.

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