Abstract Changing the concentration of cholesterol in the plasma membrane of isolated outer hair cells modulates electromotility and prestin-associated charge movement, suggesting that a similar manipulation would alter cochlear mechanics. We examined cochlear function before and after depletion of membrane cholesterol with methyl-β-cyclodextrin (MβCD) in an excised guinea pig temporal bone preparation. The mechanical response of the cochlear partition to acoustic and/or electrical stimulation was monitored using laser interferometry and time-resolved confocal microscopy. The electromechanical response in untreated preparations was asymmetric with greater displacements in response to positive currents. Exposure to MβCD increased the magnitude and asymmetry of the response, without changing the frequency tuning of sound-evoked mechanical responses or cochlear microphonic potentials. Sodium salicylate reversibly blocked the enhanced electromechanical response in cholesterol depleted preparations. The increase of sound-evoked vibrations during positive current injection was enhanced following MβCD in some preparations. Imaging was used to assess cellular integrity which remained unchanged after several hours of exposure to MβCD in several preparations. The enhanced electromechanical response reflects an increase in outer hair cell electromotility and may reveal features of cholesterol distribution and trafficking in outer hair cells.

Keywords Methyl-β-cyclodextrin (MβCD) · Salicylate · Interferometry · Time-resolved confocal microscopy · Prestin-associated charge movement · Filipin · Hair cell · Membrane · Electrical stimulation · Intramembranous charge movement · Guinea pig · Cochlea · Hearing · Aging

Abbreviations

OHC Outer hair cell
MEM Minimum essential medium
MβCD Methyl-β-cyclodextrin
NaSal Sodium salicylate

Introduction

Outer hair cell (OHC) electromotility contributes to the movements of the cochlear partition [20, 36] consistent with a presumed role of OHCs in counteracting the effects of viscous damping [4, 6]. A membrane-based motor in the OHC lateral wall is responsible for electromotility [6, 8, 9, 13, 25, 29]. This motor converts the energy in the transmembrane electric field directly into mechanical energy and can generate forces at frequencies approaching 80 kHz [19]. Prestin (SLC26A5) is an integral membrane protein found in OHC membranes [2, 5, 33, 56, 57] and is an important component of the membrane motor [31]. Prestin introduces a reactive component to the total membrane charge movement that is phase-shifted relative to the ohmic charge through ion channels [50]. This reactive or
displacement current is often used as an experimental measure of prestin function.

We have previously shown that membrane cholesterol content affects prestin-associated charge movement in both OHCs [42] and human embryonic kidney (HEK) 293 cells expressing prestin [42, 46, 49]. A hyperpolarizing shift and decrease in the total amount of the prestin-associated charge moved is linearly related to increases in membrane cholesterol [46]. Cholesterol comprises a substantial portion of eukaryotic cell membranes, accounting for 35% to 45% of membrane lipids [3, 12]. Cholesterol distribution in the plasma membrane varies and in many cells its concentration is elevated in membrane rafts. The OHC is unusual in that its lateral wall plasma membrane contains less cholesterol than its basal or apical membranes and conventional rafts are not observed [7, 18, 37, 38, 42, 44].

Cholesterol dependent changes in OHC function would be expected to modulate cochlear function, specifically cochlear electromechanics. Increases and decreases of membrane cholesterol affect the production of distortion product otoacoustic emissions [42] consistent with membrane cholesterol playing a role in cochlear mechanics. Further support for a role in cochlear function comes from the fact that OHC membrane cholesterol concentration decreases during OHC development [42] over the same time frame as the onset and maturation of OHC electromotility [5, 23]. In this study, we examine the effect of depleting membrane cholesterol on electrically and/or acoustically evoked movements of the cochlear partition in a well-characterized excised temporal bone preparation.

Materials and methods

Animal preparation

All animal procedures used in this study were approved by the local ethics committee (permit N460/09). Albino guinea pigs of both sexes, weighing 250–400 g, were anesthetized, decapitated, and the temporal bone quickly removed and fixed in a custom-made holder. The bulla was gently opened, followed by submersion of the preparation in a well-characterized excised temporal bone preparation.

Oxygenated MEM. The perfusion system kept the preparation vital for up to 4 h and was also used to deliver pharmaceuticals. Measurements of vibrations and electrical potentials were made through a second opening in the otic capsule over scala vestibuli in turn 4 near the cochlear apex (Fig. 1b). The apical opening served as an exit for the perfusate and was also used to advance microelectrodes into scala media (Fig. 1b). All experiments were carried out at room temperature (~21°C).

Recordings typically began approximately 25 min after decapitation when the preparation still had a positive endocochlear potential (EP) of 10–30 mV. The EP decreased with time and was approximately 0 mV 1 h after decapitation. In order to monitor the condition of the preparations, we repeatedly measured the amplitude of the cochlear microphonic potential at different frequencies.

Interferometry

A custom-made interferometer [27] equipped with a 25× water immersion lens [35] was used to measure the acoustically and electrically evoked movements of the organ of Corti. Application of artificial reflectors was not required and the noise floor of the system was well below the measured vibration amplitudes. For acoustic stimulation, we used a windowed 200 ms five tone complex, avoiding the frequencies of potential distortion products. A stepping protocol was used for the electrical stimulation. Each recording began with a 50-ms interval at 0 μA; the current was then stepped to the desired value (1–30 μA) and held constant for 100 ms, followed by a step back to the 0 μA level (Fig. 1c). Charge buildup in scala media was avoided by placing a second 100-ms-long current pulse of opposite polarity outside of the measurement window.

Time-resolved confocal microscopy

Hair cells were stained with RH795 (Invitrogen, The Netherlands; [17] delivered through the perfusion system at the beginning of the experiment. An upright laser scanning microscope (LSM 510, Zeiss, Germany), equipped with a 40× water immersion lens (0.8 NA, Zeiss, Germany), was used to acquire a continuous series of 37 images. Each series required approximately 40 s at the frame size and scanning speed used. A pure tone was used for acoustic stimulation and a square wave of approximately 5 Hz and 50% duty cycle was used for electrical stimulation. Hence, the electrical stimulus alternated between positive and negative current during the image acquisition, which was locked to the acoustic and electric stimuli, as described previously [26]. For every pixel in the image series the phase of the acoustic stimulus and of the electrical stimulus were known. Using Fourier rows, images
for positive and negative current stimulation were reconstructed at 12 equally spaced acoustic phases between 0 and 2\(\pi\). Motion was estimated based on the computation of the optical flow between images of consecutive phases [21].

Electrodes

Borosilicate electrodes were freshly pulled, filled with an endolymph-like solution (1.3 mM NaCl, 31 mM KHCO\(_3\), 23 \(\mu\)M CaCl\(_2\), 128.3 mM KCl; pH=7.4;0.30 osM/kg) and beveled to an impedance of approximately 2 M\(\Omega\). Microphonic potentials were measured with an IX1 amplifier (Cornerstone, Dagan Corp., USA, equipped with 10× head stage) and digitized with a 16-bit A/D board (National Instruments, USA) or a signal analyzer (35665A, Hewlett-Packard, USA). The current stimulator (A395, World Precision Instruments, USA) was controlled by custom software (LabVIEW, National Instruments, USA).

Cholesterol depletion and salicylate administration

Baseline cochlear microphonics, acoustic, and electrically evoked movements were measured in the untreated temporal bone preparation until stable repeatable values were obtained. Membrane cholesterol was depleted by adding 1 mM methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD, Sigma) to the oxygenated MEM perfusing the temporal bone, and the measures were repeated. In three preparations, the M\(\beta\)CD perfusate was rinsed from the reservoir and MEM containing 10 mM of NaSal was added. Perfusion was continued until a change in the electromechanical response was observed at which time the NaSal perfusate was rinsed and replaced with normal MEM.

Results

Exposure to M\(\beta\)CD enhances the electrical–mechanical response

Glass electrodes positioned in scala media were used to inject electrical currents (Fig. 1). In response to positive current, which depolarizes outer hair cells, Hensen’s cells moved toward scala vestibuli, the +10 \(\mu\)A current step in Fig. 2a evoking a 30-nm position shift. This position shift closely followed the input waveform, although the onset and offset of the mechanical responses were slower than the command voltage applied to the constant current stimulator. Having reached the plateau, the amplitude remained stable...
for the duration of the current step. After applying 1 mM of MβCD, which depletes membrane cholesterol, the magnitude of the position shift increased to 125 nm (Fig. 2b). In addition, a slow displacement increase was observed with the Hensen’s cells continuing to move toward the scala vestibuli until the end of the current step. On average, the magnitude of the position shift increased from $56 \pm 9$ nm to $163 \pm 23$ nm during MβCD perfusion (mean ± S.E.M., $n=9$; $p=0.0003$ by the Wilcoxon rank sum test).

Figure 2c shows that the step amplitude was stable before MβCD application. The increase of the response was evident minutes after its application, and the maximal increase generally occurred after 20–30 min. In several preparations, the electrically evoked responses started declining thereafter, a change reflected as an increased standard error for the late time points in Fig. 2c. Control amplitudes in the absence of MβCD were stable, although a tendency to a decrease toward the end of the recording period was evident (Fig. 2c, lower thick line). The stability of current-evoked responses in the absence of MβCD is evident from the small standard errors of the normalized control amplitudes throughout the experiment.

Responses to negative currents were also altered by MβCD perfusion. In the absence of the drug, a 10-μA current produced an ~20-nm position shift directed at scala tympani (Fig. 2d), which increased to 60 nm after 20 min of MβCD perfusion (Fig. 2e). A slow drift in position was evident also in this recording, but this effect persisted beyond the duration of the current step. Note that Hensen’s cells did not return to their baseline position at the end of the current step but rather overshoot it by some 10 nm, a pattern seen in several preparations. The time course of changes in response magnitude was similar for positive and negative currents (compare Fig. 2c and f). On average, negative current responses increased from $39 \pm 9$ to $89 \pm 10$ nm due to removal of membrane cholesterol ($p=0.006$).

To ascertain whether the kinetics of electrically evoked mechanical responses was affected by cholesterol depletion, the data were fit with a low-pass filtered version of the command voltage driving the constant current generator. In the case shown in Fig. 2a, the time constant was 5.4 ms with an amplitude of 33 nm. The time constant became slightly slower (7.3 ms, Fig. 2b) during MβCD perfusion and as noted above, the amplitude increased by a factor close to 4. Due to the slowly changing position during the plateau, we could not fit this part of the response using this simple function alone. Overall, the response kinetics showed no significant change due to MβCD perfusion (before MβCD,
outer hair cells are embedded in a matrix of supporting cells, a change in the stiffness of those surrounding structures will result in an altered electromechanical response. In the absence of an endocochlear potential, sound-evoked mechanical responses are largely determined by the stiffness, mass, and friction of the cochlear structures [32]. An indirect but useful measure of these parameters can be obtained by measuring sound-evoked mechanical responses in the absence of current. Figure 4a shows the mechanical response to a sound stimulus containing five frequencies centered on the best frequency of the recording location. The waveform acquired during MβCD perfusion is similar to the control, except for a minor increase in the noise level. Spectral analysis (Fig. 4b) using the Fourier transform corroborates this impression: Although small changes occur at some frequencies, these are within the noise floor. Aside from a slight shift towards higher frequencies, the cochlear microphonic potential also shows little change (Fig. 4c). Minor increases in cochlear microphonic potentials were seen in some preparations during MβCD perfusion, and small decreases were noted in others. Overall, MβCD did not affect the amplitude or tuning of these potentials. The data shown in Fig. 4a–c indicate that the passive mechanics of the organ of Corti are unaffected by MβCD, and the lack of change in cochlear microphonics is evidence that forward transduction is impervious to the reduction in cholesterol in the cell bodies of outer hair cells.

Figure 4 demonstrates how exposure to MβCD can result in an increase of the acoustically evoked response in the presence of a positive current. Positive current, which restores the endocochlear potential in the excised preparation, often results in larger acoustically evoked responses than either no current or negative current and MβCD could greatly enhance the increase. There was considerable variation in the increase observed following MβCD. In some cases, there would be no increase even with a major enhancement of the electromotile response. In other preparations, there was a modest increase when MβCD was

**Mechanical and electrical response is similar before and after MβCD exposure**

A possible explanation for the increased electrically evoked responses shown in Figs. 2 and 3 is a reduction in the stiffness of the organ of Corti. Because the force-generating outer hair cells are embedded in a matrix of supporting

![Image](https://placehold.it/150x150)

**Fig. 3** The magnitude of the response to positive current is greater than the response to negative current and salicylate blocks the electromechanical response. **a** Summary plot of the ratio of the magnitude of the response to positive current to the magnitude of the negative current for nine preparations. **b** Response of a representative preparation to current steps of different magnitude before and after perfusion with MβCD. **c** Electromechanical responses over time from a representative preparation to +10-μA current injections showing typical increase in response to MβCD perfusion followed by a rapid decline in response to perfusion with 10 mM NaSal.
Figure 4 shows another example of increased cochlear amplification following MβCD perfusion. Time-resolved confocal imaging and optical flow computation [22, 26] was used to measure sound-evoked motions. Acoustically evoked motions were nearly perpendicular to the reticular lamina during negative current injections, with a peak amplitude of approximately 300 nm (black trajectory in Fig. 5a). Positive current increased perpendicular vibrations to ~360 nm (gray trajectory in Fig. 5a). The parallel motion component, directed along the horizontal axis of the image, appears more responsive to positive current and consequently shows a larger increase than the vertical component.

After 10 min of MβCD perfusion at 1 mM, negative current vibrations were unchanged, as seen by comparing the black trajectories in Fig. 5a, b. The peak amplitude remained close to 300 nm, and the major axis of the trajectory remains in the same orientation. Thus, during negative currents, sound-evoked vibrations are quite stable and not significantly affected by MβCD. However, sound-evoked responses during positive current were increased. Note that the amplitude increases more for movements parallel to the reticular lamina giving the trajectory a more elliptic shape (gray trajectory in Fig. 5b). This is an important change, as it would be expected to be more effective in deflecting hair cell stereocilia.

The cellular components of the cochlear partition remain intact following MβCD exposure.

Short-term perfusions with MβCD (<1 h) did not produce obvious morphological changes in the organ of Corti, as evidenced by confocal imaging of the measurement site after loading cells with the fluorescent membrane dye RH795. In Fig. 6a, note that Reissner’s membrane retains its normal honeycomb configuration and that supporting cells near the measurement site all appear intact. The cell membranes of supporting cells were clearly labeled, and the lipid droplets inside Hensen’s cells are also visible. Fragmentation of lipid
droplets, a common sign of acute cellular stress in the cochlear apex, was not observed. On focusing deeper into the organ of Corti (Fig. 6b), it is seen that outer and IHC appear normal with clearly delineated cell membranes showing no signs of swelling or other structural abnormalities. The section through the organ of Corti is oblique and the full length of the OHC bodies cannot be inspected in this image. However, preparations with long-term exposure (>1 h) to MβCD frequently, but not always, showed pathology, most commonly OHC shrinkage.

The antifungal macrolide filipin is highly fluorescent and binds specifically to membranes containing cholesterol and can therefore be used for visualizing such membranes. It was used to image the organ of Corti in control samples (Fig. 6c) as well as samples treated with MβCD (Fig. 6d). While there was a decrease in staining intensity in MβCD-treated samples, labeling was still observed in all cell types in the organ of Corti, confirming that removal of cholesterol with MβCD is incomplete. Preparations that were not exposed to filipin had autofluorescence that was at least one order of magnitude smaller than the fluorescence intensity observed in filipin treated samples (Fig. 6c, d). The MβCD-induced decrease in intensity should be interpreted with caution given the difficulty in quantifying fluorescent labels such as filipin.

Discussion

The site of action is the outer hair cell

The increase in the electromechanical response observed on exposure to MβCD most likely results from the depletion of cholesterol from the plasma membrane of OHCs. We have previously reported on how membrane cholesterol alters prestin-associated charge movement in prestin-transfected HEK cells. Decreasing membrane cholesterol shifts prestin-associated charge movement towards more depolarized membrane potentials and increased the charge density [46].
During cochlear development, the concentration of cholesterol in the OHC lateral wall decreases [37, 42] while the subsurface cisternae appear and mature [55]. At the same time, there is an increase in OHC electromotility [5, 23] accompanied by a depolarizing shift in prestin-associated charge movement and an increase in its charge density [1, 40]. The increase in the cochlear electromechanical response we observe resembles the developmental increase in OHC electromotility that is concurrent with a decrease in plasma membrane cholesterol. Sodium salicylate has long been known to block OHC electromotility [15, 28, 48]. The reversible reduction of the βCD-enhanced response with sodium salicylate further supports the site of action of βCD as the OHC lateral wall plasma membrane.

The absence of an βCD-dependent change in cochlear microphonics implies that membrane cholesterol depletion was confined to cells exposed to scala tympani perfusion including the portion of Reissner’s membrane from the helicotrema to the recording site. Filipin labeled the exposed cells, and cochlear microphonics remained unchanged indicating Reissner’s membrane was functionally intact. The continuation of an electromechanical response is further testimony to the continued integrity of Reissner’s membrane because its damage results in an altered impedance that would alter the electromechanical response.

Implications of the βCD response increase on cholesterol trafficking in the OHC

Measuring OHC reactive charge movement simultaneously with electromotile length changes reveals that the voltage dependence for normalized length changes is quantitatively identical to the voltage dependence of the normalized reactive charge movement [24, 53]. This supports the use of the reactive, prestin-associated current as equivalent to electromotility. Exposure to βCD increases the electromechanical response to positive current by approximately four times (Fig. 2c) which is more than twice what would be expected from the effect of cholesterol depletion on prestin-associated charge density [46].

Immunohistochemical labeling suggests that prestin is uniformly distributed along the OHC lateral plasma membrane [56]. Prestin function, however, is not uniform; it is non-functional at the synaptic pole [25] and has a non-uniform distribution along the lateral wall [52]. Mechanically evoked prestin-associated charge movement is maximal midway between the cuticular plate and the nucleus. A plausible explanation for the non-uniform distribution would be a cholesterol concentration gradient that reaches a minimal value in the middle of the cell. This cholesterol concentration gradient is apparent in photomicrographs of filipin-stained OHCs [37] and is consistent with the lower fluorescence we observe in the OHC region of Fig. 6c. The “specific” prestin-associated charge movement would be maximal in the region with the least cholesterol and decrease with increasing cholesterol concentration [46] towards either end. In addition, the voltage at maximum gain for the charge movement will hyperpolarize as cholesterol increases further reducing prestin function at normal holding potentials. The net result is that the prestin residing towards the ends of the OHC would appear to be non-functional. The non-uniform distribution of cholesterol in normal OHCs would lower the whole-cell electromechanical response to less than what it would be if the cholesterol concentration were uniformly low. βCD exposure would reduce the cholesterol concentration towards the level at the middle of the cell and the total “whole-cell” charge density would increase by more than a factor of two, consistent with the observed increase in cochlear electromechanics (Fig. 2c).

The mechanism by which cholesterol is maintained at a low concentration in the lateral wall membrane is not known but is likely to involve lipid trafficking with the subsurface cisternae. Membrane turnover occurs at the apex and the base of the OHC [30, 38], and the newly added membrane would be expected to have a normal eukaryotic plasma membrane cholesterol concentration. Cholesterol will diffuse from membrane recently enriched by exocytosis at the base and apex of the OHC and, in the absence of any other contribution to cholesterol trafficking, the concentration will equilibrate throughout the basolateral plasma membrane. If the subsurface cisternae actively sequester cholesterol from the plasma membrane, the cholesterol concentration will decrease until it reaches a minimum value near the middle of the lateral wall. Both immunohistochemical [56] and immunogold [34] labeling of prestin in OHCs indicate that prestin density is less in the basal pole than in the lateral wall. The reduced prestin may be the result of prestin recycling which has been shown to increase with deglycosylation and increased cholesterol [43]. Prestin recycling cannot occur in the lateral wall because the cortical lattice and subsurface cisternae prevent vesicular trafficking. The reduced prestin may contribute to the absence of prestin function observed in the synaptic pole of the OHC.

Implications for hearing health

Serum dyslipidemia The impact of serum dyslipidemia on cardiovascular health is well-known, and heart disease is a major public health concern. There is little information as to how serum dyslipidemia might lead to alterations in the lipid composition of cell membranes. The mechanisms underlying cellular membrane cholesterol homeostasis have been studied for many years and involve a variety of regulatory feedback pathways within cells. The relation between serum dyslipidemia and hearing is further confounded by the limited
vascularization of the organ of Corti (presumably to minimize cardiovascular pressure changes so that we do not hear our pulse). There are reports of a correlation between serum dyslipidemia and hearing loss [16, 41, 47] that are balanced by other reports finding no correlation. Two animal studies have suggested that hearing problems associated with age or resulting from a lipid challenge are ameliorated with statins [11, 51]. Systemic administration of a β-cyclodextrin has been used to treat animal models of Niemann–Pick type C disease. The treatments generally resolve the major problems of the disease, but a non-reversible hearing loss was observed in cats [54]. The hearing loss is consistent with the loss of distortion product otoacoustic emissions we observed with MβCD [42]. Hair cell membranes are buffered by a variety of processes involved in cholesterol trafficking when dyslipidemia is treated with statins. These are bypassed with the β-cyclodextrin approach, suggesting that care must be taken to not be too aggressive in attempting to modulate membrane cholesterol in the ear.

Aging Aging is associated with a drop in endocochlear potential [39, 45] which may be linked to conventional atherosclerotic cholesterol involvement reducing perfusion of the stria vascularis. The low endocochlear potential is mimicked in our study by the no current condition. The cochlear electromechanical response is increased either by restoring the endocochlear potential with a positive current or by reducing membrane cholesterol levels. Our findings suggest that restoring the endocochlear potential and decreasing membrane cholesterol may improve some forms of geriatric hearing loss.

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