Chlorpromazine alters cochlear mechanics and amplification: \textit{in vivo} evidence for a role of stiffness modulation in the organ of Corti

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Abstract

While prestin-mediated outer hair cell (OHC) electromotility provides mechanical force for sound amplification in the mammalian cochlea, proper OHC stiffness is required to maintain normal electromotility and to transmit mechanical force to the basilar membrane (BM). To investigate the in vivo role of OHC stiffness in cochlear amplification, chlorpromazine (CPZ), an antipsychotic drug that alters OHC lateral wall biophysics, was infused into the cochleae in living guinea pigs. The effects of CPZ on cochlear amplification and OHC electromotility were observed by measuring the acoustically and electrically-evoked BM motions. CPZ significantly reduced cochlear amplification as measured by a decline of the acoustically-evoked BM motion near the best frequency (BF) accompanied by a loss of nonlinearity and broadened tuning. It also substantially reduced electrically-evoked BM vibration near the BF and at frequencies above BF (up to 80 kHz). The high frequency notch (near 50 kHz) in the electrically-evoked BM response shifted towards higher frequency in a CPZ concentration-dependent manner with a corresponding phase change. In contrast, salicylate resulted in a shift in this notch towards lower frequency. These results indicate that CPZ reduces OHC-mediated cochlear amplification, probably via its effects on the mechanics of the OHC plasma membrane rather than via a direct effect on the OHC motor, prestin. Through modeling, we propose that with a combined OHC somatic and hair bundle forcing, the upward-shift of the ~50 kHz notch in the electrically-evoked BM motion may indicate stiffness increase of the OHCs that is responsible for the reduced cochlear amplification.

Keywords: chlorpromazine, outer hair cell, basilar membrane, electromotility, stiffness
Introduction

In the mammalian cochlea, outer hair cells (OHCs) possess a unique motor capability termed “electromotility” whereby they change the somatic length in a voltage-dependent manner (Brownell, 1985; Santos-Sacchi, 1991). The electromotility is assumed to provide mechanical force to the basilar membrane (BM), therefore locally amplifying the sound-evoked traveling wave in the cochlea to ensure normal cochlear sensitivity (Dallos, 1992; 1996). While OHC electromotility depends on the motor protein prestin (Zheng et al., 2000; Dallos and Fakler, 2002; Liberman, 2002), proper stiffness of OHCs is also essential to the electromotile capability (Kakehata and Santos-Sacchi, 1995; Santos-Sacchi et al., 2001). OHC stiffness is largely dependent on the biophysical properties of the basolateral wall, which has a unique nanoscale organization of three layers: the plasma membrane, the cortical cytoskeleton, and the subsurface cisterna (Dallos, 1992; Brownell et al., 2001). The static axial stiffness of isolated OHCs has been intensively investigated (e.g., Holley and Ashmore, 1988; Zenner et al., 1992; Hallworth, 1995; Iwasa and Adachi, 1997; Ulfendahl et al., 1998), and in vitro data have associated OHC stiffness with OHC electromotility (e.g., Russell and Schauz, 1995; Hallworth, 1997; Dallos et al., 1997; Chan, et al., 1998; He and Dallos, 1999; Lue and Brownell, 1999; Oghalai et al., 2000; He et al., 2003; Batta et al., 2003; Borko, et al., 2005). However, the in vivo role of OHC stiffness in electromotility and cochlear amplification in the intact organ of Corti (OoC) has remained unclear.

Manipulation of OHC stiffness in the in vivo experiments is difficult, but data from in vitro work suggest that at least three lines of experiments could be considered for in vivo study: (1) Applying electrical stimulation to the cochlea to modulate OHC stiffness. (2) Manipulating OHC turgor by changing perilymph osmolarity. (3) Applying pharmacological agents into the cochlea that could alter OHC stiffness. Modulation of
BM mechanical tuning and amplification by DC current has been observed (Parthasarathi et al., 2003). However, the putative stiffness changes were not verified. Manipulation of perilymph osmolarity can modulate cochlear sensitivity (Oghalai et al., 2006), but this method is not specific to the target (OHCs), and many other variables (e.g., mass, viscosity, and damping) are affected which complicates the analysis. The effects of several pharmacological agents (i.e., salicylate, diamide, ocadaic acid, and chlorpromazine) on OHC lateral wall mechanics have been reported (Dieler et al., 1991; Russell and Schauz, 1995; Adachi and Iwasa, 1997; Hallworth, 1997; Lue and Brownell, 1999; Oghalai et al., 2000; Lue et al., 2001; Borko et al., 2005). In particular, chlorpromazine (CPZ), an antipsychotic drug that affects plasma membrane biophysics, alters OHC lateral wall micromechanics and electromotility without a known direct action on prestin (Oghalai et al., 2000; Lue et al., 2001). CPZ preferentially intercalates into inner leaflet of the phospholipid bilayer (Sheetz and Singer, 1974; Leu et al., 2001), so that causes an inward bend of the plasma membrane. This curvature alteration results in decrease of the fluidity of the plasma membrane, which is associated with the tension or stiffness of the OHC lateral wall (Oghalai et al., 2000). Unlike salicylate (SAL) which also causes a curvature change (but outward bend) of the plasma membrane and reduces OHC electromotility by a direct inhibition effect on prestin (McLaughlin, 1973; Kakehata and Santos-Sacchi, 1996; Oliver et al., 2001), CPZ changes the OHC length-voltage relation without an effect on the absolute value of OHC length change, and this effect is likely solely through an effect on the OHC plasma membrane biophysics (Oghalai et al., 2000; Morimoto et al., 2002; Lue et al., 2001; Oliver et al., 2001). Therefore, CPZ appears a relatively ideal chemical for investigation of the in vivo role of OHC stiffness modulation.

To test the hypothesis that in vivo OHC electromotility is related to OHC stiffness, we investigated the effects of CPZ on both acoustically and electrically-evoked BM
motions. While the acoustically-evoked BM motion provides a direct measure of the cochlear amplification (Robles and Ruggero, 2001), the electrically-evoked BM motion represents the OHC-mediated electro-mechanical transduction process, thus providing a tool for investigation of the \textit{in vivo} OHC electromotility (Nuttall and Ren, 1995; Grosh et al., 2004). In addition, the high frequency (i.e., up to 100 kHz) electromotile responses of BM evoked by bipolar current stimulation across the cochlear partition in the basal turn is likely an asymmetrical local resonance associated with OHCs (Grosh et al., 2004), and could be in a mode of piezoelectric resonance (Spector et al., 2003; Weitzel et al., 2003; Scherer and Gummer, 2004). Thus, investigation of the electrically-evoked high-frequency responses of BM may inform about the cochlear electromotile processes and mechanical properties that can not be obtained with acoustic stimulation (Grosh et al., 2004). In this report, we present data from living guinea pigs showing CPZ-induced alterations of cochlear mechanics, along with evidence that stiffness changes in the organ of Corti play a role in modulation of cochlear amplification.

\textbf{Materials and Methods}

\textbf{Animal preparation.} Pigmented guinea pigs (strain 2NCR, obtained from Charles River Laboratory) weighing 250-350 g were used (n=32). The animals were housed in American Association for Accreditation of Laboratory Animal Care-approved facilities. Experimental protocols were approved by the Institutional Animal Care and Use Committee, Oregon Health & Science University. The animals were anesthetized using both ketamine (40 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). Supplemental doses of both anesthetics were given on a schedule or as needed, judging by leg withdrawal to a toe pinch.
Rectal temperature of the animals was maintained at 38±1°C with a servo-regulated heating blanket. Cochlear temperature was additionally controlled by supplemental heat to the head from a lamp and a heated head-holder. The guinea pig’s head was firmly fixed in the head-holder, which was mounted on a custom-made manipulator and was electrically isolated from the operation table. A tracheotomy was performed and a ventilation tube was inserted into the trachea to insure free breathing. A ventral and post-auricular combined approach was used to open the left auditory bulla and expose the cochlea. The middle ear muscle tendons were carefully sectioned.

Cochlear sensitivity was monitored throughout the experiment by recording the evoked compound action potential (CAP) of the auditory nerve. For this purpose, a ball electrode made of Teflon coated silver wire (75 µm in diameter) was placed in the round window niche. An Ag/AgCl wire was inserted into neck soft tissue to serve as the ground electrode. A plastic coupler with two speakers (made of 1/2" B&K microphones) mounted inside was fitted to the ear canal to deliver acoustic stimuli. Tone bursts (10 ms in duration, 1 ms rise/fall, 2-36 kHz) were delivered to the ear canal to evoke the CAP. The round window signal was amplified 1000 times and was displayed on an oscilloscope for CAP threshold assessment. In addition, the quadratic distortion signal at 900 Hz from the round window evoked by two tones at 18 and 18.9 kHz was repeatedly checked during the surgery on the cochlea. This distortion product is highly sensitive to surgical trauma at the location corresponding to 18 kHz.

**Measurement of basilar membrane velocity.** The magnitude and phase of BM transverse velocity were measured at the location corresponding to the 18 kHz best frequency (BF) using a laser interferometer (Polytec OFV 1102). To measure BM motion, a small fenestra was created in the first-turn scala tympani bony wall of the
cochlea. Gold coated glass beads (10-30 µm in diameter) were put onto the BM at the appropriate location in the OHC region to serve as reflective objects that track the motion of the BM. A cover slip was used to close the hole to prevent perilymph surface vibration and to correct the optical distortion of imaging through a fluid meniscus. A laser beam from the laser interferometer was focused on a bead with the aid of a compound microscope (Nuttall et al. 1991). Velocity of stapes motion was measured in the end of some experiments by measuring the motion of a gold coated glass bead (150 µm in diameter) on the footplate of stapes. The data of stapes velocity were used to normalize the BM motion.

For measurement of acoustically-evoked BM motion, acoustic stimuli (3-24 kHz, 10-100 dB SPL) were generated by a Tucker-Davis Technologies (TDT) System II and presented to the external ear canal through a speaker in the acoustic speculum. The output signal of the interferometer is proportional to the velocity of the targeted bead. This signal was low-pass filtered by an 8-pole filter with a 40 kHz cut-off frequency digitized by a 16 bit A/D converter. BM velocities were determined after fast Fourier transform (FFT) of the Hanning-windowed responses from the interferometer. Phases of BM motion in reference to TDT output signal were also measured. The reference phase was that of the electrical output to the speaker. The data were stored on a PC hard disk for offline analysis.

Electrically-evoked BM responses provide indirect measures of in vivo OHC electromotility (Nuttall and Ren, 1995; Grosh et al., 2004). Techniques for measurement of electrically-evoked BM velocity responses have been reported previously (Grosh et al., 2004). Briefly, as illustrated in Figure 1, two wire electrodes (Pt–Ir, 50 µm in diameter) were inserted into the scala vestibuli (SV) and scala tympani (ST) respectively in the basal turn, forming a bipolar pair at the BM measurement location across the
cochlear duct. Sinusoidal current from a constant-current stimulator was delivered through the electrodes. The BM velocity in response to sinusoidal current stimuli was measured with the same laser interferometer as described above. Voltage control to the constant-current stimulator was from the oscillator output of a digital lock-in amplifier (Stanford Research Systems SR830). The current intensity was 100 µArms and the frequency range was 5-80 kHz. Signals from the laser interferometer were recorded by the same lock-in amplifier and displayed in terms of amplitude and phase.

**Perilymphatic perfusion of the scala tympani.** Perilymphatic perfusion was performed to deliver pharmacological agents into the scala tympani of the cochlea. An inlet hole (diameter ~ 70 µm) was made in the scala tympani close to the round window niche and the hole for BM measurement served as fluid outlet (Figure 1). A three-way perfusion device that allows solution substitution was used for scala tympani perfusion. A polyethylene tube was connected to this device and its fine tip (diameter ~ 60µm) was inserted into the inlet hole of the cochlea. Tissue glue was applied to seal the inlet hole and fasten the tube in position. Agents in artificial perilymph were infused into the scala tympani at a rate of 2 µl/min using a syringe pump (WPI, SP 210iw). The normal artificial perilymph composition is: NaCl 132 mM, KCl 3.5 mM, NaHCO₃ 25 mM, CaCl₂ 1.3 mM, MgCl₂ 6H₂O 1.14 mM, NaH₂PO₄ H₂O 0.51 mM, Tris 5.0 mM, Glucose 3.3 mM, Urea 2.1 mM. The pH of all solutions was adjusted to ~7.4 and the osmolarity was 300±10 mOsm.

Artificial perilymph perfusion using the technique described here did not affect the BM response. However, because the artificial perilymph was used to carry the agents for intra-cochlear perfusion and also for drug washout, BM measurement after artificial perilymph perfusion was always performed in each experiment as control, although we also measured the BM motion before perfusion. The duration of perfusion for each agent
was usually 10 minutes but was prolonged to as long as 30 minutes in certain cases as needed. The duration for drug washout was usually 30 minutes. Measurement of BM motion was usually performed at least five minutes after the end of each perfusion. Effluent from the outlet hole was absorbed within the bulla using cotton wicks.

**Statistical analysis**

An analysis of variance (ANOVA) was utilized to determine significant difference across treatment groups. A probability of $< 0.05$ is considered a statistically significant difference.

**Results**

*Effects of CPZ on acoustically-evoked BM vibration*

In cochlear mechanics research, the BM responses are usually presented in the form of “mechanical tuning curve” by plotting out the BM vibration magnitude in response to a certain stimulus level as a function of frequencies. A whole set of normal BM mechanical tuning curves to acoustic stimuli ranging from 3 to 24 kHz at 10-100 dB SPL (10 dB/step) is presented in Figure 2 A. Also, calculation of the gain of BM motion from the ratio of BM velocity/stapes velocity provides information of cochlear amplification. Figure 2 B shows an example of the transfer functions of the BM velocity gain (for sound levels 10-100 dB SPL) in a sensitive ear. The tuning of BM responses is sharp and the gain of BM is large at low sound levels. They become less sharp and smaller respectively when the sound level is increased, exhibiting a nonlinear “compressive growth” of the tuning curves (Robles and Ruggero, 2001). Scala tympani perfusion of
CPZ substantially affects both the mechanical tuning and the gain of BM motion. As shown in a typical animal in Figure 2, when 5 mM CPZ was administered, the magnitude of BM motion in response to low-level acoustic stimulation near the best frequency (BF, i.e., the frequency where the BM exhibits the highest response) was greatly reduced (Figure 2, C). The mechanical tuning was broadened with a shift of the BF towards lower frequency. The compressive growth of responses to higher-level sound was less obvious compared to normal responses (compare panels A and C in Figure 2). A loss of the gain of BM motion by at least 30 dB was observed in this example (Figure 2, D). These changes demonstrate a loss of cochlear amplification under CPZ administration.

More details of alterations in BM motion induced by CPZ are shown in Figure 3. In an example shown in Figure 3 A, significant reduction in the peak magnitude of the mechanical tuning curve (evoked by 40 dB SPL tones) indicates a loss of sensitivity near BF, while a broadened tuning indicates a loss of frequency specificity. A shift of BF towards lower frequency is also obvious, which is a feature of sensitivity loss. Figure 3 B shows the phase change of BM motion as a function of frequency in the same animal. With CPZ perfusion, the phase lags below BF but leads above BF compared with the phase in control. To learn whether or not CPZ also affects the passive mechanics of BM, we compared the BM responses to very high sound level (100 dB SPL) in conditions with and without CPZ. As shown in another example in Figure 3 C and D, 10 mM CPZ perfusion significantly reduced the magnitude of BM response evoked by acoustic stimulation at 100 dB SPL. Phase changes similar to that with low level sound stimulation were also observed (compare Figure 3 B and D).

The sensitivity loss induced by 5 mM CPZ was approximately 20 dB (Figure 3 E, data from six animals). Calculation of the $Q_{10\ dB}$ value (the BF divided by the bandwidth of the tuning curve 10 dB from the tip, a measure of the sharpness of tuning) shows significant difference in tuning ($p<0.01$) between control ($5.69 \pm 1.34$, mean $\pm$ SD, n=6)
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and 5 mM CPZ (3.29 ± 1.03, mean ± SD, n=6). These results indicate a worse tuning in BM motion due to CPZ. Loss of nonlinearity of BM motion was also seen as shown by BM velocity magnitude input-output functions (Figure 3 E).

In pilot experiments, different concentrations of CPZ (i.e., 0.1-50 mM) were used, and concentration-dependent effects of CPZ on BM motion were observed (data not shown). However, CPZ at 0.1 mM had a very minimal effect. At 1.0 mM, a clear effect could be seen, yet the reduction of BM vibration at BF was still very small (approximately 5 dB). A significant effect on BM motion was observed when CPZ concentrations were 5 mM and 10 mM, and its effects were reversible after washout with artificial perilymph (see an example in Figure 3 F). The reversible effects verified that alterations in BM responses we observed were due to the effects of CPZ. Above 15 mM, CPZ severely suppressed cochlear sensitivity, which could not recover. For this reason, we used 5 mM and 10 mM on most of the animals in this study. In addition, because of the concern that CPZ could act on OHC via an effect on cholinergic receptors (Park et al., 2001), strychnine (100 µM), a potent cholinergic receptor antagonist, was infused into the scala tympani before the application of CPZ. The effect of CPZ was not blocked by strychnine, ruling out the possibility of an action on OHC cholinergic receptors (data not shown).

**Effects of CPZ on electrically evoked BM vibration**

In sensitive cochleae, electrically-evoked BM responses in the OHC region near the 18 kHz location had sharply-tuned multiple peaks below 20 kHz (“below-BF-part”) which corresponded to traveling waves (see the phase change with frequency in Figure 4 B), and poorly-tuned responses above 20 kHz (“above-BF-part”) with a notch near 50 kHz (Figure 4 A). This notch was always accompanied by a phase change (Figure 4 B) as we reported previously (Grosh et al., 2004). CPZ affects the electrically-evoked BM
responses in a concentration-dependent manner. The effects on the below-BF-part were similar to those of acoustically-evoked BM vibration (Figure 4 A and C). In the above-BF-part, a reduction in the magnitude of BM vibration was also seen. But, the more interesting change was the frequency shift of the ~50 kHz notch towards higher frequency when CPZ was applied (Figure 4 A). The amount of frequency shift was CPZ concentration-dependent, and was as large as several kilohertz (in Figure 4 A, it was 3.8 kHz with 5 mM CPZ and up to 5.4 kHz with 10 mM CPZ). The frequency of phase change corresponding to the notch systematically shifted accordingly (Figure 4 B). The change in the phase at the notch frequency indicates a likely change in the mode of vibration of the OoC (Grosh et al., 2004), and thus these data suggest that the OoC vibration mode at this frequency is altered by CPZ. With CPZ concentrations at and below 10 mM, the effects of CPZ on the electrically evoked BM responses were reversible (see Figure 4 C for a recovery in BM vibration magnitude in the below-BF-part, and the return of the high-frequency notch after washout).

Since the phase changes due to CPZ in the acoustically-evoked BM motion (Figure 3) are in accordance with the stiffness increase as proposed by other’s work (Cooper and Guinan, 2001), the frequency shift of the ~50 kHz notch towards higher frequency in the electrically-evoked BM responses with CPZ perfusion (Figure 4) may imply stiffness increase of OHCs by the action of CPZ on the plasma membrane. To test this hypothesis, we applied salicylate (SAL), a chemical that blocks the motor protein of OHCs and has been shown to reduce the in vitro OHC stiffness (Shehata et al., 1991; Kakehata and Santos-Sacchi, 1996; Hallworth, 1997; Lue and Brownell, 1999; Oliver et al., 2001) with its effects on the plasma membrane biomechanics resulting in an outward bend in the curvature of plasma membrane, a change being opposite to that of CPZ (McLaughlin, 1973; Gutknecht and Tosteson, 1973). SAL caused a shift of the high-frequency notch towards lower frequency, which is opposite to the shift caused by CPZ.
This “downward” shift was small in degree but unambiguous, and was reversible with artificial perilymph washout (Figure 5 A, B).

**Modeling the notch and effect of CPZ in the organ of Corti**

To investigate the relationship between the above-BF notch in electrically evoked BM responses and OHC stiffness, a simplified phenomenological model of the organ of Corti (OoC) was developed and then its results were compared with our physiological data.

In this model, electromotility was hypothesized to arise from two sources: a piezoelectric effect in the OHC soma (e.g., Mountain and Hubbard, 1994; Spector et al., 2003; Weitzel et al., 2003; Deo and Grosh, 2005) and a hair bundle (HB) force (e.g., Kennedy et al., 2005) proportional to the current passing through the apical pole of the OHC. As the notch frequency was well above the BF at this location, we assumed that the fluid-structure waves arising from this local excitation were evanescent. Hence these waves would be attenuated rapidly in either direction as the mode is cut off. This simplification allowed us to model the effect of the fluid and distant structures of the cochlea as a reactive load locally on the BM. Thus, our model of the cochlea consists of a single radial cross-section of the OoC. The BM is then loaded by the additional reactive impedance needed to fit the data.

Key structural components of this model are shown in Figure 6. In developing the model for the local OoC cross-section the following assumptions are made: a) For frequencies well above BF, the spring-like impedance of the HB connecting the cuticular plate (CP) and reticular lamina (RL) to the tectorial membrane (TM) is dominated by mass-like impedance of TM and that the motion of the TM is much smaller than the other masses. Note that we have made simulations where this assumption is relaxed (not
shown) and the conclusions we make from the model described herein are unchanged;
b) A lumped piezoelectric model of the OHC is used to model its electromotile behavior;
c) The connection between the basal pole of the OHC and the BM through the Deiters’
cell (DC) is achieved via a spring and damper; d) The BM is modeled as a spring-mass-
damper system.

With these assumptions the OoC model reduces to a three degree of freedom
system, as described in Figure 6. The definitions of the parameters are given in the
figure caption. The force arising from the electromotility of the OHC is given by \( F_{ohc} \). This
force is applied to mass \( M_2 \) and \( M_3 \) while the HB force \( F_{hb} \) is applied to mass \( M_3 \) and the
TM as shown in the Figure 6. Assuming time harmonic motion of the form \( \exp(j\omega t) \),
where \( j = \sqrt{-1} \) and \( \omega \) is the radian frequency of vibration, the system of equations for
the model takes the following form:

\[
\begin{bmatrix}
-M_1\omega^2 + j\omega(C_1 + C_2) + K_1 + K_2 & -K_2 - j\omega C_2 & 0 \\
-K_2 - j\omega C_2 & -M_2\omega^2 + j\omega(C_2 + C_3) + K_2 + K_3 & -K_3 - j\omega C_3 \\
0 & -K_3 - j\omega C_3 & -M_4\omega^2 + j\omega(C_3 + K_4 + K_3)
\end{bmatrix}
\begin{bmatrix}
u_1 \\
u_2 \\
u_3
\end{bmatrix} =
\begin{bmatrix}
0 \\
-F_{ohc} \\
F_{ohc} - F_{hb}
\end{bmatrix}
\]

Here \( u_1, u_2, \) and \( u_3 \) represent the displacements of masses \( M_1 \) (BM + \( \frac{1}{2} \) DC), \( M_2 \)
(\( \frac{1}{2} \) DC + \( \frac{1}{2} \) OHC), and \( M_3 \) (\( \frac{1}{2} \) OHC + RL), respectively. The motion of different structures
is simplified to be colinear, which is not really the case in the OoC. Inclusion of the
different orientations of the structures changes the stiffness matrix, but the result
remains the same qualitatively. The inclinations (angular tilt) of the different structures
were therefore not taken into account. For the OHC, a piezoelectric model is used (see
The total external force on the OHC ($F_{\text{ext}}$, tensile is positive) and current through it ($I_{\text{ohc}}$) are given by:

\begin{align*}
F_{\text{ext}} &= K_3(\Delta u_{\text{ohc}}) + \varepsilon \Delta \phi \\
I_{\text{ohc}} &= j\omega \varepsilon (\Delta u_{\text{ohc}}) + \Delta \phi / Z_{\text{ohc}}
\end{align*}

Where $\Delta u_{\text{ohc}} = u_3 - u_2$, $Z_{\text{ohc}}$ is the electrical impedance of the OHC basolateral wall ($1/Z_{\text{ohc}} = 1/R_m + j\omega C_m$), $R_m$ is the basolateral resistance, $C_m$ is the basolateral capacitance, $\varepsilon$ is the electro-mechanical coupling coefficient, and $\Delta \phi$ is the transmembrane voltage relative to the resting value. Consequently the force that the OHC applies to the mass due to electrical excitation is $F_{\text{ohc}} = -\varepsilon \Delta \phi$. Solving Eqs. (2) and (3) yields,

\begin{equation}
F_{\text{ohc}} = -\varepsilon Z_{\text{ohc}}(I_{\text{ohc}} + j\omega \varepsilon (u_3 - u_2))
\end{equation}

thus the OHC force adds terms to the left hand side of the matrix (i.e., alters the coefficient matrix) as well as to the right-hand side (current dependent terms) in Eq. 1. We make the assumption that the current passing through the OHC is equal to that provided by the constant current source, in this case a 100 $\mu$A rms sinusoidal term.

Therefore, the known forcing term in Eq. 1 is $I_{\text{ohc}} = I_0 \exp(j\omega t)$, where $I_0$ is the amplitude of the current from the constant current source. We can solve Eq. 1 for the mechanical response to a constant current source excitation.

The HB force acts at the apical pole of the OHC (see Figure 6). We assume the HB force takes the form $F_{\text{hb}} = \beta F_{\text{ohc}}$, where $\beta$ is an unknown proportionality constant. One can also assume that the HB force is proportional to $I_{\text{ohc}}$, with the same resulting conclusions (results not shown). Parameters used in the model are detailed in Table 1.
Predictions from the model are made by solving Eq. 1 using the parameters given in Table 1 subject to a time harmonic oscillatory current, $I_0$. As in the experiments the driving force is the applied current. In addition to solving for the displacement, we solve Eq. 1 in closed form for the frequencies corresponding to zeros of the BM displacement of mass 1 ($u_1$). In the limit of very small damping the “notch” frequencies is given by

$$\omega_{\text{notch}} = \sqrt{\frac{K_4 - \beta K_3}{M_3}}.$$  \hspace{1cm} (5)

Considering the case $\beta=0$ (i.e., OHC somatic forcing only), we have

$$\omega_{\text{notch}} = \frac{K_4}{M_3}.$$  \hspace{1cm} The notch frequency only depends on the HB-RL stiffness (embodied by $K_4$) and mass loading the apex of the OHC ($M_3$), but not the OHC stiffness ($K_3$).

Embellishing the model with further details (for instance, adding a stiffness coupling between the BM and the RL) does not change this conclusion. Figure 7A and 7B show model response predictions of amplitude and phase with different OHC stiffness values numerically demonstrating the theoretical result of Eq. (5). If this model is a faithful representation of the mechanics in vivo then it implies that the stiffness of the structures on the apical end of OHC, such as the HB and/or the RL, is being affected by CPZ, rather than the stiffness of the OHC soma (see Fig. 7A and 7B).

If force production by the HB is involved, this force is applied to the cuticular plate and RL as reflected in Eq. 1. The notch frequency, $\omega_{\text{notch}}$, is computed from Eq. (5), recalling that $\beta$ is the ratio of HB forcing to OHC somatic forcing in the direction perpendicular to the BM. The notch frequency here is sensitive to OHC stiffness manipulation as embodied by the dependence on $K_3$. Simulations of this model with an assumed HB forcing using $\beta = 0.05$ are shown in Figure 7C and 7D. The model shows a
shift in the notch frequency when OHC stiffness is changed, in which upward notch-frequency shift occurs with increased OHC stiffness and vice versa. With higher HB forcing (increasing $\beta$), the shift in frequency of the notch is greater, but the notch becomes less prominent. Further, with HB forcing the notch is not as deep as for the case of OHC somatic forcing alone. As shown in Figure 7C, an alteration in the stiffness of HB-RL also results in a shift in the notch frequency, just as in the somatic only forcing case.

Discussion

Cochlear amplification and OHC electromotility altered by CPZ

We demonstrate here that CPZ reduces *in vivo* OHC electromotility and thus affects cochlear amplification. A previous *in vitro* study (Lue et al., 2001) has shown that CPZ alters isolated OHC electromotility as measured by nonlinear capacitance (NLC) and voltage-dependent OHC length-change. In that study the peak capacitance ($C_{mpk}$) and OHC length-voltage transfer function shifted in a depolarizing direction, indicating an inhibitive effect of CPZ on OHC motility. In accordance with this implication, CPZ-induced elevations in CAP and DPOAE thresholds have been reported (Oghalai, 2004). Observation of the BM motion enabled us to gain better insight into the cochlear mechanics and the effectiveness of the cochlear amplifier. In this study, 5 mM CPZ substantially reduced cochlear sensitivity as evidenced by: a) a reduction in velocity magnitude of acoustically-evoked BM motion, and a loss of gain by 20-30 dB near BF (Figure 2); b) a broadening of tuning (Figures 2 C, D and 3 A); c) a reduction of nonlinearity (Figures 3 E, F). These changes are consistent with the aforementioned
reduction in electromotility of isolated OHCs (Lue et al., 2001) and alterations in CAP and DPOAEs (Oghalai, 2004). In addition, a reduction in electrically-evoked BM vibration magnitude both below and above BF (Figure 4) confirms the decrease of OHC electromotility that could account for the decline of cochlear amplification.

We considered, but discounted, the following two mechanisms that could underlie the actions of CPZ on OHC electromotility.

(1) An action via anti-dopaminergic receptor effect as summarized by Awad and Voruganti (2005). Evidently, the OHCs were not affected by this mechanism since dopamine was found to act only on the lateral efferents innervating the inner hair cells but not on the OHCs (Eybalin, 1993). In addition, the endocochlear potential (EP) was not affected by CPZ (Oghalai, 2004), ruling out an EP-related OHC driving force reduction via an effect on cochlear blood flow that could be modulated through a dopamine receptor-mediated mechanism (Ernster and Meyers, 1986; Zeng, et al., 2004).

(2) Actions on cholinergic receptors on the OHCs. Cholinergic receptor-mediated OHC electromotility has been well elucidated (e.g., Housley and Ashmore, 1991; Erostegui et al., 1994; Dallos et al., 1997; Kalinec et al., 2000). CPZ could act on certain subtypes of cholinergic receptors (Park et al., 2001) so that the observed CPZ effects could be mediated by these receptors. In the control experiment, the effect of CPZ on cochlear sensitivity was not affected by strychnine, a potent antagonist of cholinergic receptor. This possibility was thus ruled out.

The following two mechanisms for CPZ’s action are better supported by our model and data, as well as data in the literature:

(1) Alterations in the voltage-motility relationship of the OHCs. Unlike SAL (a prestin inhibitor), CPZ does not reduce the $C_{m_{pk}}$ or the absolute value of voltage-dependent OHC length change. However, CPZ shifts the voltage location of the $C_{m_{pk}}$ and OHC length-voltage transfer function in a depolarizing direction (Lue et al., 2001).
These alterations could be due to a change in surface charge of the plasma membrane (Zhang, et al., 2001). The OHCs thus may not operate at their optimal electromotility due to the change of the motor status. Reduced OHC motility will directly lead to reduced cochlear amplification.

(2) Alterations in biomechanics of the OHC lateral wall. In vitro data suggest that CPZ acts mainly on the plasma membrane of OHCs (e.g., causing inward bend, altering the tension and reducing the fluidity of the plasma membrane, see Sheetz and Singer, 1974; Leu et al., 2001; Oghalai et al., 2000) without any evidence for a direct effect on the motor protein (Oghalai et al., 2000; Lue et al., 2001; Morimoto et al., 2002). The experimental data and modeling work presented here suggest that an increase in stiffness of the OoC may occur due to CPZ (see detailed discussion below). The model results (Figure 7A and C) also show that increasing OHC stiffness reduces BM response to bipolar electric stimulation. Thus, reduced motility by CPZ could be attributable to increased stiffness of the OHCs, which is consistent with the observation of OHC motility and stiffness in an in vitro study (Borko, et al., 2005). In addition, CPZ-induced shift of the voltage for $C_{m, pk}$ could result in an OHC axial stiffness change (e.g., He and Dallos, 1999; Iwasa, 2000), which in turn will affect the motility. However, this effect leads to a reduction in OHC stiffness and thus an increase of motility. We assume this effect is only a minor one which is dominated by the stiffness increase effect of CPZ as shown by our data.

**OHC stiffness change by CPZ: experimental data and model prediction**

The most interesting and novel finding in this study is the shift of the ~50 kHz notch towards higher frequency in the electrically-evoked BM vibration when CPZ was applied (Figure 4). In acoustically-evoked BM motion, CPZ-induced phase lead above
BF (Figure 3) has suggested an increase in stiffness of the cochlear partition (Cooper and Guinan, 2001). The ~50 kHz notch shift induced by CPZ thus may be associated with the stiffness change. Indeed, while a shift of this notch towards higher frequency occurred with administration of CPZ, a shift towards lower frequency was observed when salicylate (SAL), a drug known to decrease the OHC axial stiffness (Shehata et al., 1991; Kakehata and Santos-Sacchi, 1996; Hallworth, 1997; Lue and Brownell, 1999), was applied (Figure 5). The experimental data therefore suggested that a change in the stiffness of OHCs and probably some other structures in the OoC was involved in controlling the shift of this high-frequency notch. Opposite directions of this notch shift by SAL and CPZ suggest opposite actions on biomechanics (e.g., plasma membrane curvature and stiffness) of the OHCs, which are in line with previous in vitro studies (Oghalai et al., 2000; Morimoto et al., 2002; Lue et al., 2001; Oliver et al., 2001). Thus, a shift of the ~50 kHz notch towards higher frequency may indicate an increase of the stiffness of the OoC, and vice versa.

It is noticed that SAL has less effect on the degree of ~50 kHz notch shift than CPZ, while their effects on the magnitude of BM motion are similar. These may suggest different mechanisms of their actions on the OHC electromotility. We speculate that SAL affects OHC electromotility dominantly by a direct effect on the motor protein along with a relatively minor effect on OHC lateral wall biophysics; whereas, CPZ probably affects OHC electromotility more indirectly through effects on the voltage-motility relationship as well as on the stiffness of OHC lateral wall.

The modeling work presented here supports the CPZ-induced stiffness modulation of cellular components in the OoC. Our simple model shows that if OHC somatic forcing is the only electromotile forcing present in the cochlea at these high frequencies, then CPZ can affect a shift in the notch frequency by affecting the stiffness of hair bundle (HB) and/or reticular lamina (RL) as shown in Figure 7 A and B, though
stiffness change of OHC soma has no effect on the notch frequency shift in this case. Access to these structures is possible for CPZ, which could be through an intra-cellular pathway to HB (because CPZ can penetrate the OHC plasma membrane and intercalate into the inner leaflet of this membrane, see Sheetz and Singer, 1974; Leu et al., 2001), and via extra-cellular fluid in the OoC and probably also intracellular pathway to RL.

Intriguingly, if HB motility is introduced (as proposed by Kennedy et al., 2005), our model predicts that the OHC stiffness will play a role in setting the frequency of the notch. Then CPZ-induced OHC stiffness change can cause a shift of the notch towards higher frequency as shown by predictions of the notch shift by the combined HB-OHC somatic forcing model in Eq. 5. This is consistent with the proposal by He and Dallos (1999) that relatively modest stiffness changes of OHCs might have significant influence on cochlear mechanics. Importantly, this could be in vivo evidence that OHC stiffness plays a role in determining the mode of organ of Corti motion in the face of HB motility. Again, stiffness changes of HB and RL also play a role in this case (Figure 7 C and D).

It should be pointed out that the effect of CPZ on the plasma membrane is nonspecific and thus CPZ-induced changes in the micromechanics of supporting cells in the OoC should also be taken into account. Especially, the stiffness change of Deiters’ cells should not be ignored since a prominent role for Deiters’ cells in cochlear sensitivity is reported (Flock et al., 1999). However, the stiffness of the rigid pillar cells and Deiters’ cells is largely determined by the intracellular filaments and tubules that span the distance between the RL and BM (Slepecky, 1996). Changes in plasma membrane stiffness of these cells may not significantly affect the overall stiffness of them. Indeed, our model does not show involvement of the stiffness of supporting cells \(K_2\) in the shift of the high-frequency notch.

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References


Figure legends

Figure 1. Schematic drawing illustrating intra-cochlear current stimulation, perilymphatic perfusion and basilar membrane vibration measurement. BM: basilar membrane; RW: round window; SV: scala vestibuli; ST: scala tympani. Gray arrows in the perfusion tube and cochlea show the flow of perfusate.

Figure 2. Chlorpromazine (CPZ) induced alterations in magnitude and gain of acoustically-evoked basilar membrane (BM) vibration. A and B: magnitude and gain of BM velocity at sound levels ranging from 10-100 dB SPL, with artificial perilymph perfusion serving as control. C and D: magnitude and gain of BM velocity, with 5 mM CPZ infused into the scala tympani. Numbers next to each curve indicate the sound pressure level (in dB SPL) used to evoke the BM velocity responses. The single dashed curve in panel D is plotted from the data in panel B at 10 dB SPL serving as a comparison for the loss of the gain.

Figure 3. Acoustically-evoked BM vibration altered by chlorpromazine (CPZ). A and B: BM velocity magnitude and phase evoked by 40 dB SPL pure tones. C and D: BM velocity magnitude and phase evoked by 100 dB SPL pure tones. E: Averaged input-output functions (n=6) of BM velocity at BF. F: An example of input-output functions of BM velocity at BF showing the reversible effect of CPZ. The legend “control” in all panels stands for the condition with artificial perilymph perfusion.

Figure 4. Chlorpromazine (CPZ) altered electrically-evoked BM vibration. Sinusoidal current (5-70 kHz, 100 µArms) was injected into the cochlea with scala vestibuli to scala tympani configuration. A and B: Magnitude and phase of BM velocity evoked by
electrical stimulation with 5 mM and 10 mM CPZ perfusion. Arrows in panel A indicate the notch frequency. C and D: Magnitude and phase of BM velocity evoked by electrical stimulation with 5 mM CPZ perfusion and with artificial perilymph washout. In control conditions artificial perilymph was infused.

**Figure 5.** The effect of salicylate (SAL) on electrically-evoked BM vibration. A: Magnitude of BM velocity. B: Phase of BM velocity. The high-frequency notch near 50 kHz shifted towards a lower frequency due to SAL. This effect was reversible after washout. In control conditions artificial perilymph was infused.

**Figure 6.** Schematic of the simple model to represent OoC mechanics. Masses of the basilar membrane (BM), Deiters’ cells (DCs) and outer hair cells (OHCs) are lumped together as suggested in the figure (\(M_1 = \text{Mass of BM} + 1/2 \text{Mass of DC}; M_2 = 1/2 \text{Mass of DC} + 1/2 \text{Mass of OHC}; M_3 = 1/2 \text{Mass of OHC} + \text{Mass of reticular lamina (RL)}\)). \(K_1\) and \(C_1\) are the BM stiffness and damping respectively; \(K_2\) and \(C_2\) are the stiffness and damping of DCs respectively; \(K_3\) and \(C_3\) are the stiffness and damping of OHCs respectively; \(K_4\) is the stiffness of the apical attachment of the OHC, including the hair bundle (HB), RL and other cell structures supporting the RL. \(F_{\text{ooc}}\) represents equal and opposite active forcing from OHCs due to their piezoelectric-like behavior. \(F_{\text{hb}}\) is active forcing from the HBs. \(u_1, u_2, \text{and } u_3\) represent displacement of masses \(M_1, M_2 \text{ and } M_3\) respectively.

**Figure 7.** Model response to current excitation for different OHC stiffness (\(K_3\)) and HB-RL stiffness (\(K_4\)) values. In (A, B) \(\beta = 0\) while in (C, D) a non-zero \(\beta = 0.05\) is used (representing HB forcing). The notch in the BM response is more pronounced in absence
of hair bundle forcing but also does not show any shift with OHC stiffness changes. The shift in notch frequency is larger for higher hair bundle forcing. The magnitude of BM response is much higher than in experiment. This discrepancy is because all the 100µA current is assumed to flow through only one radial cross section (a single outer hair cell) rather than spreading down the length of the cochlea.
### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius of OHC at 5mm from base (r)</td>
<td>5 (\mu)m</td>
<td>Estimated from Dallos (1996)</td>
</tr>
<tr>
<td>Length of OHC at 5mm from base (l)</td>
<td>35 (\mu)m</td>
<td>Estimated from Dallos (1996)</td>
</tr>
<tr>
<td>Mass of OHC</td>
<td>0.172 ng</td>
<td>(\text{Mass}=\frac{\rho \pi r^2 l}{16}); (\rho=1000) (density of water), factor of 16 is used to fit Frank et al. (1999).</td>
</tr>
<tr>
<td>Stiffness of OHC (K_3)</td>
<td>12 mN/m (at rest)</td>
<td>Rest value from Frank \textit{et al.} (1999); CPZ value estimated from He and Dallos (1999) data for percentage stiffness change at extreme hyperpolarized voltages.</td>
</tr>
<tr>
<td>Electromechanical coupling (\epsilon)</td>
<td>98.8 nN/V (at rest) 49.4 nN/V (with CPZ)</td>
<td>Deo and Grosh (2005)</td>
</tr>
<tr>
<td>OHC resistance (R_m)</td>
<td>25 Mohms</td>
<td>Housley and Ashmore (1992)</td>
</tr>
<tr>
<td>OHC capacitance (C_m)</td>
<td>23.28 pF (at rest) 12.64 pF (with CPZ)</td>
<td>Deo and Grosh (2005)</td>
</tr>
<tr>
<td>BM mass + Fluid loading</td>
<td>78.1 ng</td>
<td>Calculated to have BM resonance at 18kHz.</td>
</tr>
<tr>
<td>RL/HB stiffness (K_d) (Free parameter)</td>
<td>9mN/m</td>
<td>Calculated to match dip frequency around 50 kHz</td>
</tr>
<tr>
<td>Deiters Cell Stiffness (K_2)</td>
<td>1 N/m</td>
<td>Estimated from Naidu and Mountain (1998)</td>
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<tr>
<td>Deiters Cell Mass (Free parameter)</td>
<td>21.99 ng</td>
<td>Calculated to have DC resonance around 30kHz</td>
</tr>
<tr>
<td>HB Forcing factor (\beta)</td>
<td>0.05</td>
<td>Free parameter</td>
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<tr>
<td>Damping, (C_1, C_2, C_3)</td>
<td>17.6 (\mu)Ns/m, 3.3 (\mu)Ns/m, 32.1 nNs/m</td>
<td>Calculated to have damping coefficient of 1 for BM and 0.5 for OHC and DC.</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2ab
Figure 2cd
Figure 3a-b
Figure 3c-d
Figure 4a-b
Figure_4c-d
Figure 6

Cross section of the organ of Corti
Figure 7a-b
Figure 7c-d