Published in final edited form as:

Hear Res. 2016 December ; 342: 39–47. doi:10.1016/j.heares.2016.09.006.

The potential use of low-frequency tones to locate regions of outer hair cell loss

Aryn M. Kamerer^{a,*}, Francisco J. Diaz^a, Marcello Peppi^b, and Mark E. Chertoff^a

^aUniversity of Kansas Medical Center, Kansas City, KS 66160, USA

^bThe Charles Stark Draper Laboratory, USA

Abstract

Current methods used to diagnose cochlear hearing loss are limited in their ability to determine the location and extent of anatomical damage to various cochlear structures. In previous experiments, we have used the electrical potential recorded at the round window –the cochlear response (CR) – to predict the location of damage to outer hair cells in the gerbil. In a follow-up experiment, we applied 10 mM ouabain to the round window niche to reduce neural activity in order to quantify the neural contribution to the CR. We concluded that a significant proportion of the CR to a 762 Hz tone originated from phase-locking activity of basal auditory nerve fibers, which could have contaminated our conclusions regarding outer hair cell health. However, at such high concentrations, ouabain may have also affected the responses from outer hair cells, exaggerating the effect we attributed to the auditory nerve. In this study, we lowered the concentration of ouabain to 1 mM and determined the physiologic effects on outer hair cells using distortion-product otoacoustic emissions. As well as quantifying the effects of 1 mM ouabain on the auditory nerve and outer hair cells, we attempted to reduce the neural contribution to the CR by using near-infrasonic stimulus frequencies of 45 and 85 Hz, and hypothesized that these low-frequency stimuli would generate a cumulative amplitude function (CAF) that could reflect damage to hair cells in the apex more accurately than the 762 stimuli. One hour after application of 1 mM ouabain, CR amplitudes significantly increased, but remained unchanged in the presence of high-pass filtered noise conditions, suggesting that basal auditory nerve fibers have a limited contribution to the CR at such low frequencies.

Keywords

Cochlear response; Cochlear microphonic; Cumulative amplitude function; Ouabain; Compound action potential; Distortion-product otoacoustic emissions

1. Introduction

In the audiology clinic, the diagnosis of hearing loss relies heavily on the audiogram. While certain audiologic patterns differentiate sensorineural from conductive hearing loss, the audiogram is unable to discern the underlying anatomical damage in the cochlea; damage to

*Corresponding author. Department of Hearing & Speech, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA.

different structures of the inner ear can manifest as similar patterns of hearing loss on an audiogram (Suzuka and Schuknecht, 1988). Thus, specific sites-of-lesion for many sensorineural hearing losses go unidentified, leaving the audiologist or physician to speculate on treatment and prognosis. As treatment for hearing disorders advance –both technologically in terms of hearing aids and cochlear implants, as well as medically with the promise of hair cell regeneration –so does the need for more sensitive and specific diagnostic measures.

The cochlear microphonic (CM) is an electrophysiologic response to acoustic stimulation that results from current flow through ion channels in hair cell membranes, and therefore is a strong candidate to measure the health of outer hair cells (Withnell, 2001; Cheatham et al., 2011). The CM, as picked up by an electrode placed at the round window (RW), contains the responses from hair cells across the entirety of the cochlea, weighted by their distance to the electrode. This poses two potential problems: a high-frequency stimulus causes maximal displacement of the basilar membrane and thus large phase incoherence at the base of the cochlea, proximal to the electrode site, altering the amplitude of the response and risking a false diagnosis of damage (Whitfield and Ross, 1965; Laszlo et al., 1972; Patuzzi, 1987). This can be solved by using a low-frequency stimulus so that the majority of the basilar membrane remains in-phase. The response, however, is still dominated by basal hair cells near the electrode, which can confound an attempt to measure outer hair cell (OHC) health at the apex of the cochlea (Dallos, 1969; Patuzzi et al., 1989). To solve this problem, Chertoff et al. (2012, 2014) implemented a filtered noise paradigm in which 733 & 762 Hz tone stimuli were embedded in high-pass filtered noise with seventeen consecutively increasing cutoff frequencies, allowing them to measure a cumulative response from seventeen corresponding regions along the length of the cochlear partition. Taking into account the electric field decay and geometric distance from each point of measurement to the electrode, they modeled a growth function of the CM amplitude as a function of distance along the length of the cochlear partition. This growth function was deemed the cumulative amplitude function (CAF), and they showed that damage to OHCs will alter the growth of this function (Chertoff et al., 2012, 2014).

Although the CAF proved a fairly accurate predictor of the location of onset of anatomical damage to OHCs ($r = 0.734$, $p = 0.001$), there were two outstanding obstacles to the predictive power of the 762 Hz CAF. The first is that the literature suggests that a significant proportion of the RW response is generated from auditory nerve potentials in addition to OHCs, especially for low-frequency stimuli (Henry, 1995; Patuzzi et al., 1989; He et al., 2012; Lichtenhan et al., 2014). In order to quantify the proportion of neural and OHC potentials present in the RW response, or cochlear response (CR), Chertoff et al. (2014) damaged the auditory nerve with 10 mM of an ototoxic drug, ouabain, and recorded the CR before and after an acute application to the RW niche. Chertoff et al. (2014) reported reductions in CR amplitude to a 762 Hz tone of up to 70% (at low signal levels) after the application of 10 mM ouabain on the RW for 30 min. They attributed the effect primarily to the loss of basal auditory nerve fibers; however, they also found a small decrease in distortion-product otoacoustic emission (DPOAE) amplitudes to a 24 kHz primary tone, indicating some OHC damage.

Ouabain is $\text{Na}^+\text{-K}^+\text{-ATPase}$ pump inhibitor and has a high affinity for the $\alpha 3$ -receptor subunit isoform (O'Brien et al., 1994; Pierre et al., 2008) which, in the cochlea, is expressed by auditory nerve fibers (McLean et al., 2009). Several studies found that the sensitivity to ouabain is dependent on nerve type: type I neurons are more sensitive than type II (Lang et al., 2005), and low spontaneous rate fibers are more sensitive than high spontaneous rate fibers (Bourien et al., 2014). Ouabain can be used to selectively damage the auditory nerve because of its low affinity for the $\alpha 1$ -receptor isoform found in the epithelial cells of the cochlea, including hair cells (McGuirt and Schulte, 1994). Several studies argue over the effect of ouabain on OHCs, which seems to be dependent on species, dose, time, and method of application. Bourien et al. (2014) measured the physiologic effects of ouabain on OHCs by recording DPOAEs after an acute application of 100 μM ouabain on the gerbil RW for 30 min. They found that ouabain did not affect the DPOAE amplitudes in the f_2 range of 0.5–20 kHz at 60 and 55 dB SPL signal levels for f_2 and f_1 , respectively. Schmiedt et al. (2001) showed similar results for 1 mM ouabain, but found that 10 mM significantly reduced DPOAE amplitude after 3 h. In spite of the apparent hair cells' resistance to low concentrations of ouabain, Fu et al. (2012, 2013) reported detrimental anatomical effects of ouabain on rat cochlear hair cells; application of 1 mM ouabain to a dissected Organ of Corti for 24 h resulted in an 80% loss of inner hair cells and 50% loss of OHCs. Chertoff et al. (2014) found an effect on DPOAEs at high frequencies with 10 mM ouabain. In Experiment 1 of this study, we aimed to quantify the physiologic effects, via DPOAEs, of a smaller dose (1 mM) on OHCs in order to confirm the conclusion that the large drop in CR amplitude found by Chertoff et al. (2014) was indeed a consequence of auditory nerve fiber loss and not OHC dysfunction.

In addition to the 762 Hz response containing large amounts of activity originating from the auditory nerve, another issue in using the 762 Hz CAF to predict location of OHC loss was the shape of the growth curve itself. The CAF resulting from CR recordings at each high-pass filtered noise condition to 762 Hz creates a sigmoidal curve that has a shallow, if non-existent, slope at low cutoff frequencies, which could make any anatomical changes in the apical regions of the cochlear partition difficult to diagnose. Fig. 1a shows a schematized typical 762 Hz CAF curve (solid line) and how the curve would change due to damage in apical regions (dashed line). Since the normal curve has little growth in the apex, damage to this region could be missed because the effect on the resulting CAF is not pronounced. In Experiment II, we hypothesized that a stimulus lower in frequency than 762 Hz would result in a steeper growth in the apical regions of the CAF. Fig. 1b shows the hypothesized low-frequency CAF with a steeper growth in amplitude from the apical region of the cochlear partition (solid line), which, when damaged, would result in a more dramatic change to the CAF curve (dashed line). With this in mind, we recorded the CR to near-infrasonic stimuli (45 & 85 Hz) with the intent of both circumventing phase-locking activity arising from the low-frequency tails of basal fiber tuning curves –resulting in a CR representing hair cell activity –as well as produce a CAF that would grow in a manner such that OHC damage in the apex could be more apparent. The purpose of this study, therefore, was to find a stimulus that would avoid a neural contribution, in order to more purely measure OHC health; and provide a CAF curve that would be more sensitive to physiologic changes in the apex.

2. Experiment 1: materials and methods

DPOAEs were obtained from ten Mongolian gerbils (*Meriones unguiculatus*) weighing between 40 and 70 g. Prior to surgery animals were sedated with an intraperitoneal injection of pentobarbital (64 mg/kg), and maintained with hourly supplemental intramuscular injections of one-third the initial dose. The depth of sedation was monitored by heart-rate and pulse-oxygen levels every 10–20 min (MouseOx, Starr Life Sciences, Oakmont, PA). Internal temperature was maintained at 37° C with a heating pad (Harvard, Holliston, MA) and monitored with a rectal probe. The bulla was exposed by removing the surrounding muscle and tissue, and a pick was used to open a small hole over the RW niche. Condensation and excess fluids were absorbed with a cotton wick. CAPs presented in Fig. 2 from Chertoff et al. (2014) were recorded from a silver-wire ball electrode placed in the RW niche using a micromanipulator, and a needle electrode in the hind limb muscle to serve as ground. All experimental procedures were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee.

2.1. Stimuli and physiologic recordings

DPOAEs were recorded to 21 msec tones with f_1 primary frequencies of 1, 2, 4, 8, 16, and 24 kHz, with an f_2/f_1 ratio of 1.23. The tones were delivered via earbuds (Sony) attached to an ER10B microphone, and coupled to a tube which was inserted into a plastic ring glued to the bony portion of the ear canal. The frequency response of the microphone was flattened with an FIR filter in order to correct for poor high-frequency fidelity. Emissions were recorded to increasing f_1 levels of 10–70 dB SPL in 5 dB increments, while maintaining f_1 levels 10 dB greater than f_2 . The microphone recorded the time-domain signal and Fourier analysis determined the amplitude of the cubic difference tone (CDT; $2f_1 - f_2$) for each stimulus level. We recorded CAPs in a previous experiment (see Chertoff et al., 2014) to 1, 2, 4, 8, 16, and 24 kHz tone bursts. Stimuli were generated, presented, and recorded as described in Chertoff et al. (2014).

2.2. Procedure

The animals were designated to either a control or experimental group. Once the animals were sedated and the bulla opened, DPOAEs were collected for baseline and also to determine whether the animals had existing hearing loss. If DPOAE amplitudes were two standard deviations below normative data collected by Earl and Chertoff (2012), the animal was considered hearing impaired and was not used in the study. Healthy animals then received either the control or experimental treatment. Animals in the experimental group received 1 mM ouabain dissolved in artificial perilymph (AP): 120 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl_2 , 5.5 mM glucose, and 20 mM HEPES, with an adjusted pH of 7.5 via NaOH. The control group received AP only. Approximately 1–2 μL of either solution was dripped into the RW niche. After 30 min, the niche was dried with a cotton wick. Thirty minutes post-wick, DPOAEs were recorded again. The same experimental procedure was used in Chertoff et al. (2014) to record CAPs.

2.3. Statistical analysis

To determine the effect of ouabain on the growth of DPOAEs, the mean threshold across animals was determined as the signal level at which the DPOAE amplitude consistently increased with signal level. The DPOAE amplitude was regressed as a function of signal level for each frequency, as shown in the following model,

$$y_i = b_0 + b_1 Level + b_2 Level^2 + b_3 Group + b_4 (Level \times Group) + b_5 (Level^2 \times Group) + \epsilon$$

where y is the DPOAE CDT amplitude (dB SPL), i is the stimulus frequency, b is the regression coefficient, $Level$ is the f_1 amplitude (dB SPL) for DPOAEs, $Group$ is represented with 0 for AP-treated animals and 1 for ouabain-treated animals, ϵ is the error for the model, and b_0 was a random intercept accounting for subjects heterogeneity. This polynomial model was fit using maximum likelihood estimation and then underwent backward elimination of terms that were deemed nonsignificant ($p > 0.05$), until all remaining terms in the model were significant. The regressed lines of the DPOAE growth functions at each frequency were compared across treatment conditions to determine the effect of ouabain on the growth of DPOAEs. The SPSS software was used for model fit (StataCorp LP, College Station, TX). The same model was used for CAPs, though all signal levels were included in the analysis (as opposed to above-threshold only used in the DPOAE analysis), where y is the CAP amplitude (μV) measured as the positive peak preceding N1 to the N1 minimum, and $Level$ is the stimulus signal level (dB SPL).

3. Experiment 1: results

The CAP data presented in Fig. 2 are taken from Fig. 4 of Chertoff et al. (2014) in order to clarify the conclusions drawn regarding the effect of 1 mM ouabain on the auditory nerve. CAP amplitudes and thresholds for 1, 2, 4, 8, 16, and 24 kHz were recorded before and 1 h post-application of ouabain. There were no significant differences between the groups before treatment and no significant differences between before and after application of AP-only. Therefore, the after-treatment conditions were regressed and compared between ouabain and AP-only. High-frequency CAPs (8, 16, & 24 kHz) were virtually absent after 1 mM of ouabain, while low-frequency CAPs (1, 2, & 4 kHz) amplitudes were significantly reduced.

While CAP amplitudes dropped markedly, DPOAEs suffered minimal change. Again, only the after-treatment amplitudes of ouabain and AP-only were regressed, as there were no significant differences between groups before treatment nor between before and after AP-only (Fig. 3). Threshold differences between treatment groups were <5 dB across all stimulus frequencies. The growth functions were then regressed from the mean threshold across animals. There were no significant interactions between treatment and f_1 level at f_1 frequencies of 8 kHz and below. There were, however, significant interactions between treatment and f_1 level at 16 kHz ($p = 0.015$) and 24 kHz ($p = 0.017$), indicating a reduction in DPOAE amplitude after application of ouabain at high frequencies.

4. Experiment 2: materials and methods

The eighteen animals from which we collected CAPs in Chertoff et al. (2014) were also used to record the CR either immediately prior to or post-CAP recording. Preparation of these animals was identical to that of Experiment 1, with the addition of a silver-wire ball electrode placed on the RW using a micromanipulator, and a needle electrode in the hind limb muscle to serve as ground.

4.1. Stimuli and physiologic recording

The second aim of this study was to determine if low-frequency stimuli would 1) result in a CAF curve sensitive to changes in the apex, and 2) reduce the contribution of auditory nerve fibers to the CR, which we found comprised a considerable portion of the CR at 762 Hz (Chertoff et al., 2014). We hypothesized that 1) the 45 Hz CAF curve would grow more steeply in apical regions of the cochlear partition compared to that of the 762 Hz, and 2) basal fibers would not respond to low frequencies presented at moderate sound pressures and thus the CR would be comprised mostly of signals from hair cells. The shape of the auditory tuning curve of the gerbil is unknown below 100 Hz (Schmiedt and Zwislocki, 1978), thus we were forced to estimate the frequency range needed to avoid, or significantly reduce, basal neural activity. Forty-five and 85 Hz tone bursts with 70 msec durations and 15 msec rise/fall times (TDT BioSig) were presented 50 times at a rate of 5/second to a 12 cm diameter speaker residing outside the acoustic chamber, coupled to a tube which entered the acoustic chamber and terminated at the external auditory meatus. The length of the tube enhanced resonance of the low-frequency stimuli and avoided electrical artifact. Broadband filtered noise, generated by a real-time signal processor (TDT, RZ6) and filtered with seventeen high-pass cutoff frequencies, was presented simultaneously with the stimulus via a speaker (TDT, MF1) placed approximately 3 mm from the ear canal. The frequency response of the speaker was corrected by an FIR filter such that the spectrum of the noise was flat from 0.1 to 60 kHz. Noise cutoff frequency was converted to distance along the cochlear partition from the apex (in mm) using the gerbil frequency-place map created by Müller (1996). A ¼ in. Bruel and Kjaer microphone was placed in front the ear canal to monitor sound pressure levels and signal fidelity. The CR was recorded to 45 and 85 Hz tones presented at both 80 and 90 dB SPL, sampled at 200 kHz (TDT, RZ6), amplified 50× (Stanford), filtered from 0.03 to 30,000 Hz, and again amplified (100×) and low-pass filtered from 3 kHz (Stewart).

4.2. Procedure

In order to determine whether the 45 and 85 Hz stimuli did not elicit a significant neural response, a comparison of the CR with and without the auditory nerve was necessary. Data were collected from the right ears of eighteen animals that were divided randomly into control (AP-only) and experimental (ouabain) groups (n = 9), after normal hearing sensitivity was determined via CAPs. The CR was collected before drug manipulation, then 1 mM ouabain or AP was applied and wicked after 30 min, then after a 30 min recovery period the CR was collected again, in order to observe changes due to treatment.

4.3. Statistical analysis

The CR time-domain signal was windowed with a Hanning window and converted to the frequency domain using a Fast Fourier Transform (MATLAB, Mathworks). The magnitude of the peak at the fundamental frequency (45 or 85 Hz) was considered the amplitude of the CR. A three-way ANOVA comparing both frequency (45, 85 Hz), signal level (80, 90 dB SPL), and treatment group (control, ouabain) was conducted on the amplitude of the CR in the absence of filtered noise (i.e. the whole-cochlea response).

The amplitude of the CR at each filtered noise condition was divided by the amplitude of the CR in the absence of noise, creating a CAF curve that was normalized to the CR amplitude in the absence of noise. This allowed us to compare CAF curves between animals and across frequency and signal level. The CAF curve is a curvilinear function. We used the MATLAB curve-fitting tool to find the lowest order polynomial linear regression function to fit the data and STATA was used for variable selection. Because of the sigmoidal shape of the curve, a third order polynomial was an appropriate fit to the data. The full model is shown below,

$$y_i = b_0 + b_1d + b_2d^2 + b_3d^3 + b_445Hz + b_585Hz \\ + b_6(d \times 45Hz) + b_7(d \times 85Hz) + b_8(d^2 \times 45Hz) \\ + b_9(d^2 \times 85Hz) + b_{10}(d^3 \times 45Hz) + b_{11}(d^3 \times 85Hz) \\ + \varepsilon$$

where y is the CAF, d is the distance from the apex (in mm) along the cochlear partition, b is the regression coefficient, $45Hz$ and $85Hz$ are represented as 1 for that frequency, and 0 otherwise, so that the equation for 762 Hz was represented when both $45Hz$ and $85Hz$ were equal to 0. Then backward elimination was applied to the full model to reach a final model that held only terms that were significant ($p < 0.05$). In order to determine the effects of 1 mM ouabain on the CAF curve, we introduced treatment as an interacting variable to the model, similar to that of the model shown for DPOAEs and CAPs.

5. Experiment 2: results

5.1. The effect of frequency on the cumulative amplitude function

One issue with the CAF curve created by recording the CR to 762 Hz tones is the limited growth in amplitude of the CR from apical regions of the cochlear partition (Chertoff et al., 2014). We hypothesized that a lower frequency stimulus would result in a steeper growth of the CAF from apical regions, thus we compared the CAF curves to 45 and 85 Hz with those of 762 Hz. The 45 and 85 Hz CAF data from all animals are shown in Fig. 4 alongside the data obtained by Chertoff et al., 2014.

The final model represented the CAF-distance relationship significantly ($F = 962.22$, $df = 19$, $p < 0.0001$), with each remaining interaction term being significant ($p < 0.05$) as well as the distance coefficients ($p < 0.01$) when all other predictor variables were controlled, indicating that the shape of the CAF curve was dependent on stimulus frequency. The proportion of the variance explained by the model was high ($R^2 = 0.925$), even when the

large number of parameters was accounted for ($R^2_{\text{adj}} = 0.924$). A likelihood ratio test resulted in a significant effect of frequency on the shape of the CAF curve at both 80 dB SPL ($\chi^2 = 361.43$, $df = 22$, $p < 0.0001$) and 90 dB SPL ($\chi^2 = 401.43$, $df = 21$, $p < 0.0001$) signal levels. Post-hoc pairwise comparisons of the polynomial co-efficients further indicated that the CAF obtained with the three frequencies at each signal level were significantly different from each other (Wald test, $p < 0.0001$) with the exception of 45 and 762 Hz at 90 dB SPL (Chi-squared = 0.28, $df = 1$, $p = 0.6$).

5.2. The effect of ouabain on the low-frequency cochlear response

In a previous study using 762 Hz stimulus tones, we found that a significant portion of the response originates from the auditory nerve. In this study we aimed to find a stimulus that would avoid this neural contribution –a stimulus that could more specifically be used to measure OHC health. The CR amplitudes in the absence of noise –where the entirety of the cochlea was available to respond – were compared between group means of pre-post difference in amplitude and after application of either 1 mM ouabain or AP-only. A three-way ANOVA revealed a significant interaction between signal level (80, 90 dB SPL), frequency (45, 85 Hz), and treatment group (ouabain, control; $F = 19.68$, $p < 0.001$). When frequencies were analyzed separately, we found a significant interaction between level and treatment group for 45 Hz ($F = 8.03$, $p = 0.012$), meaning that the effect of ouabain on the CR amplitude was affected by the stimulus level: for 45 Hz at 80 dB SPL, the application of 1 mM ouabain decreased the CR amplitude, but at 90 dB SPL, ouabain resulted in an increase in amplitude. We did not find such an interaction between treatment and level for 85 Hz, as the application of ouabain caused a slight increase of CR amplitude in the absence of filtered noise at both levels (Fig. 5). This is in contrast with the results from Chertoff et al. (2014), who used a 762 Hz stimulus and found a significant decrease in the CR amplitude.

In addition to the CR amplitudes in the absence of filtered noise, we compared the effects of 1 mM ouabain on the CAF (i.e. filtered noise conditions) by modeling the pre-post change as a function of distance along the cochlear partition (Fig. 6). A mixed linear regression model –identical to the model described for comparing the effects of frequency on the CAF, but with treatment as the qualitative variable of interest (instead of frequency) –was fit to the difference scores of the control and ouabain groups at each frequency and signal level separately. There were no significant effects of ouabain on the CR for any frequency or signal level. These results contrast greatly with the dramatic reduction seen in CR amplitude at 762 Hz, as shown by Chertoff et al. (2014).

6. Discussion

Ouabain is a $\text{Na}^+\text{-K}^+\text{-ATPase}$ pump inhibitor which has an affinity for the receptor subunits found in the auditory nerve, and thus has been used in physiology studies to block nerve activity. Chertoff et al. (2014) used 10 mM ouabain to quantify the proportion of the 762 Hz CR originating from the auditory nerve by comparing the CR amplitudes before and after application of the drug. They claimed a significant portion of the CR amplitude at low signal levels was from the basal nerve activity because of a large drop in CR amplitude seen after ouabain treatment. Schmiedt et al. (2001) showed that 10 mM ouabain reduces DPOAE

amplitude, indicating an effect on OHC function, which would also cause a decrease in CR amplitude. However, Chertoff et al. (2014) did not find significant effects on DPOAEs, except at 24 kHz with moderate signal levels. In order to mitigate the possible effects of ouabain on OHCs, we reduced the concentration to 1 mM, which studies found did not affect DPOAEs (Schmiedt et al., 2001; Bourien et al., 2014). We tested DPOAEs and CAPs using six probe frequencies and a large range of signal levels before and after application of either ouabain or AP.

6.1. The physiologic effects of ouabain on the auditory nerve

As expected, 1 mM ouabain had a significant effect on basal auditory nerve fibers. The CAP was virtually absent above 8 kHz, and there was a significant reduction in CAP amplitude even in apical regions (1 kHz). The tonotopic gradient of the effect of ouabain is likely due to the site of application being the RW and the short diffusion time allowed. When the diffusion time was increased from one to 3 h, there was a negligible difference in the effect on CAPs, but a noticeable increase in morbidity and complications due to prolonged sedation (data not shown). Interestingly, in the lower frequencies (1–4 kHz) where the CAP was reduced but still present, threshold remained unaffected. This confirms the findings of Schmiedt et al. (2001) and may be explained by the relationship between percentage of nerve fiber loss and effects on CAP amplitude and threshold. Bourien et al. (2014) found that the critical number of synapses that must be lost in order to increase CAP threshold was three to five times greater than the loss required to reduce CAP amplitude. It is likely that, because we did not perfuse the cochlea and tested these animals only 1 h post-application, the majority of the nerve fibers in the apex were not affected by ouabain.

6.2. The physiologic effects of ouabain on outer hair cells

Surprisingly, we found that 1 mM ouabain had similar effects on DPOAEs as 10 mM. We did not show any significant changes in DPOAE amplitude or thresholds below 8 kHz, but a significant decrease at 16 and 24 kHz in response to moderate signal levels (50–70 dB SPL). We hypothesized that 1 mM would not have an effect on DPOAEs, in conjunction with findings of Schmiedt et al. (2001) and Bourien et al. (2014), however, we did find a significant reduction at high frequencies. Upon closer inspection, our post-ouabain DPOAEs were reduced an equal amount to those presented in Fig. 4 of Schmiedt et al. (2001). The differences in our findings compared to other studies may be due to the short recovery time allowed in our experiment. It is possible that the DPOAEs could return to normal levels if allowed several hours or days to recover. Regardless, for the purposes of Experiment 2, it was important to know if any effects we found on the CR could be attributed to a change in OHC function.

6.3. The low-frequency cumulative amplitude function

One reason for recording the CR to such low-frequency tones was to attempt to increase the steepness of the CAF curve in the low-cutoff noise conditions which represent responses from primarily the apex of the cochlea. A steeper growth in the apical section of the CAF curve, as schematically shown in Fig. 1b, would have provided more opportunity to show any potential damage to this region, as a drop in amplitude would be more apparent. While statistically, the CAF curves between 45, 85, and 762 Hz were significantly different at both

80 and 90 dB SPL, with the exception of 45 & 762 Hz at 90 dB SPL, the steepness of the growth in the apical regions remained unaffected by frequency. We determined that damage to apical regions of the cochlea would not be readily apparent in the 45 or 85 Hz CAF as with the 762 Hz CAF. It is possible that even lower frequencies than those used in this study would result in a steeper slope in apical regions of the cochlear partition.

6.4. The neural contribution to the low-frequency cochlear response

Chertoff et al. (2014) concluded that a significant proportion of the CR to 762 Hz tones originated from basal auditory nerve fibers, due to the fact that 10 mM ouabain caused the CR to drop to 30% of its original amplitude at 50 dB SPL. Chertoff et al. (2014) used 10 mM ouabain, which, in gerbils, has been shown to affect the stria vascularis and significantly reduce the endocochlear potential (EP) shortly after an acute RW application (Konishi and Mendelsohn, 1970; Bosher, 1980; Schmiedt et al., 2001). It is known that a change in the EP will affect the CM (Honrubia and Ward, 1969) and perfusion of the scala vestibuli of guinea pigs with 10 mM ouabain was shown to completely wipe out the CM after only 20 min (Kuijpers et al., 1967). Therefore, it is possible that our previous conclusions regarding the extent of a neural contribution to the CR were exaggerated by a change in the EP. In this study, we reduced the concentration to 1 mM, which has a short, recoverable effect on the EP (Schmiedt et al., 2001).

1 mM ouabain resulted in a slight increase in the CR amplitude of the 45 and 85 Hz in the absence of noise. The increase in CR amplitude in this condition may be a result of a change in the EP, possibly causing the OHCs to adjust the operating point of their transducer curves to a more optimal position, increasing the amplitude of the CR. Although DPOAEs were affected only at high frequencies, it does not rule out a significant change in the EP, as OHCs are able to quickly adapt to changes in the EP (Mills et al., 1993; Lang et al., 2005). That is, because of a shift in operating point, the stimulus would act in the linear portion of the OHC transducer curve, consequently reducing DPOAEs while maintaining CM amplitudes. A more likely cause of the increase in CR amplitude is the loss of basal neural fibers reduces phase interference between the auditory nerve potentials and OHC receptor currents.

The CR was also recorded in the presence of filtered noise with increasing cutoff frequencies. The purpose of this noise was to mask responses from the auditory nerve and suppress OHC responses from specific regions of the cochlear partition. Increasing the cutoff frequency of the filtered noise with each recording allowed us to obtain a response from increasing regions of the cochlear partition (Chertoff et al., 2012, 2014) and create a growth curve known as the CAF. Contrary to the CR in the absence of noise, 1 mM ouabain had no effect on the CAF. For these two contradictory results to reconcile, the CAF –which is normalized to the CR amplitude in the absence of noise –must have increased slightly at all cutoff frequencies equal to the increase seen in the whole-cochlea CR. With respect to a neural contribution, this finding suggests that either the filtered noise effectively masked basal neural activity and the CR obtained at low cutoff frequencies (e.g. 350 Hz) indeed originated only from OHCs and perhaps apical neurons; or basal neurons do not respond to 45 & 85 Hz. Since the CR amplitude after ouabain did not decrease in the absence of noise, the latter is likely the case. Our intent, in using low-frequency tones, was to obtain a CR

with little-to-no neural contamination so that we could further our research of using the CR to locate regions of OHC loss. We conclude that the CR to 45 and 85 Hz tones does not contain basal neural activity and may be a more appropriate stimulus for the assessment of OHC health than higher frequencies.

7. Conclusions

The first experiment quantified the effects of an acute application of 1 mM ouabain on the auditory nerve and outer hair cells. Ouabain resulted in a significant reduction to the CAP amplitudes, more so at high frequencies due to the rate of diffusion of the drug in cochlear fluids. Thus we concluded that basal neural fibers were damaged and did not contribute to the CR in Experiment 2 for those animals subjected to ouabain. In terms of the effect of ouabain on outer hair cells, the drug did not result in changes to DPOAEs except at high frequencies. The isoform of the ouabain receptor present in OHCs makes them highly resistant to the drug; however, it is possible that OHCs near the site of application were affected by ouabain.

Experiment 2 used low-frequency stimuli to record the CR in order to 1) develop a CAF curve that could reflect damage to apical regions of the cochlear partition, and 2) avoid the neural contribution found using higher frequencies (762 Hz; Chertoff et al., 2014) to more specifically assess outer hair cell health. The CAF curves of 45, 85, and 762 Hz were modeled with a third-order polynomial to compare the shape of the curve between frequencies. While statistically, there were differences between frequencies, there did not seem to be a steeper growth in the apical regions using the low-frequency stimuli and thus, using the CAF to determine damage to the apex remains difficult. In terms of the effect of ouabain on the low-frequency CR, the drug did not reduce the CR amplitude, but rather tended to increase it in the absence of noise, or have no significant effect in the presence of filtered noise. This contrasted to the effect of ouabain on the 762 Hz CR which dramatically reduced the amplitude of the CR in both noise and the absence of noise (Chertoff et al., 2014). While the 762 Hz CR was reduced by the loss of the basal auditory nerve, the 45 and 85 Hz CR was not, suggesting that the basal auditory nerve does not contribute significantly to the low-frequency CR and thus, these near-infrasonic stimulus frequencies might be preferential to more specifically assess the health of outer hair cells.

Acknowledgments

This study was supported by the National Institute on Deafness and other Communication Disorders of NIH, Grant No. DC011096.

Abbreviations

CR	cochlear response
CM	cochlear microphonic
CAF	cumulative amplitude function
AP	artificial perilymph

CAP	compound action potential
DPOAE	distortion-product otoacoustic emissions

References

- Bosher SK. The effects of inhibition of the strial Na⁺-K⁺-activated ATPase by perilymphatic ouabain in the Guinea pig. *Acta Oto Laryngol.* 1980; 90(3–4):219–229.
- Bourien J, Tang Y, Batrel C, Huet A, Lenoir M, Ladrech S, Wang J. Contribution of auditory nerve fibers to compound action potential of the auditory nerve. *J Neurophysiol.* 2014; 112(5):1025–1039. DOI: 10.1152/jn.00738.2013 [PubMed: 24848461]
- Cheatham MA, Naik K, Dallos P. Using the cochlear microphonic as a tool to evaluate cochlear function in mouse models of hearing. *J Assoc Res Otolaryngol JARO.* 2011; 12(1):113–125. DOI: 10.1007/s10162-010-0240-5 [PubMed: 20957507]
- Chertoff ME, Earl BR, Diaz FJ, Sorensen JL. Analysis of the cochlear microphonic to a low-frequency tone embedded in filtered noise. *J Acoust Soc Am.* 2012; 132(5):3351–3362. DOI: 10.1121/1.4757746 [PubMed: 23145616]
- Chertoff ME, Earl BR, Diaz FJ, Sorensen JL, Thomas MLA, Kamerer AM, Peppi M. Predicting the location of missing outer hair cells using the electrical signal recorded at the round window. *J Acoust Soc Am.* 2014; 136(3):1212–1224. DOI: 10.1121/1.4890641 [PubMed: 25190395]
- Dallos P. Comments on the Differential-Electrode HE content of this paper is probably well known. *J Acoust Soc Am.* 1969 Jun.45:999–1007.
- Earl BR, Chertoff ME. Mapping auditory nerve firing density using high-level compound action potentials and high-pass noise masking. *J Acoust Soc Am.* 2012; 131(1):337–352. DOI: 10.1121/1.3664052 [PubMed: 22280596]
- Fu Y, Ding D, Jiang H, Salvi R. Ouabain-Induced cochlear degeneration in rat. *Neurotox Res.* 2012; 22(2):158–169. DOI: 10.1007/s12640-012-9320-0 [PubMed: 22476946]
- Fu Y, Ding D, Wei L, Jiang H, Salvi R. Ouabain-induced apoptosis in cochlear hair cells and spiral ganglion neurons in vitro. *BioMed Res Int.* 2013; 2013:628064.doi: 10.1155/2013/628064 [PubMed: 24228256]
- He W, Porsov E, Kemp D, Nuttall AL, Ren T. The group delay and suppression pattern of the cochlear microphonic potential recorded at the round window. *PloS One.* 2012; 7(3):e34356.doi: 10.1371/journal.pone.0034356 [PubMed: 22470560]
- Henry KR. Auditory nerve neurophonic recorded from the round window of the Mongolian gerbil. *Hear Res.* 1995; 90:176–184. [PubMed: 8974995]
- Honrubia V, Ward PH. Dependence of the cochlear microphonics and the summing potential on the endocochlear potential. *J Acoust Soc Am.* 1969; 46:388.doi: 10.1121/1.1911701 [PubMed: 5804109]
- Konishi T, Mendelsohn M. Effect of ouabain on cochlear potentials and endolymph composition in Guinea pigs. *Acta Otolaryngol.* 1970; 69(3):192–199. [PubMed: 5446886]
- Kuijpers W, Van der Vleuten AC, Bonting SL. Cochlear function and sodium and potassium activated adenosine triphosphatase. *Science.* 1967; 157(3791):949–950. [PubMed: 4378052]
- Lang H, Schulte BA, Schmiedt RA. Ouabain induces apoptotic cell death in type I spiral ganglion neurons, but not type II neurons. *J Assoc Res Otolaryngol JARO.* 2005; 6(1):63–74. DOI: 10.1007/s10162-004-5021-6 [PubMed: 15735933]
- Laszlo CA, Milsum JH, Gannon RP. Modeling and simulation of the cochlear potentials of the Guinea pig. *J Acoust Soc Am.* 1972; 52(6):1648–1660. [PubMed: 4641369]
- Lichtenhan JT, Hartsock JJ, Gill RM, Guinan JJ, Salt AN. The auditory nerve overlapped waveform (ANOW) originates in the cochlear apex. *J Assoc Res Otolaryngol JARO.* 2014; 15(3):395–411. DOI: 10.1007/s10162-014-0447-y [PubMed: 24515339]
- McGuirt JP, Schulte BA. Distribution of immunoreactive alpha- and beta-subunit isoforms of Na,K-ATPase in the gerbil inner ear. *J Histochem Cytochem Off J Histochem Soc.* 1994; 42(7):843–853.

- McLean WJ, Smith KA, Glowatzki E, Pyott SJ. Distribution of the Na,K-ATPase α subunit in the rat spiral ganglion and organ of Corti. *J Assoc Res Otolaryngol*. 2009; 10(1):37–49. DOI: 10.1007/s10162-008-0152-9 [PubMed: 19082858]
- Mills DM, Norton SJ, Rubel EW. Vulnerability and adaptation of distortion product otoacoustic emissions to endocochlear potential variation. *J Acoust Soc Am*. 1993; 94(4):2108–2122. [PubMed: 8227751]
- Müller M. The cochlear place-frequency map of the adult and developing mongolian gerbil. *Hear Res*. 1996; 94(1–2):148–156. DOI: 10.1016/0378-5955(95)00230-8 [PubMed: 8789820]
- O'Brien WJ, Lingrel JB, Wallick ET. Ouabain binding kinetics of the rat alpha two and alpha three isoforms of the sodium-potassium adenosine triphosphate. *Arch Biochem Biophys*. 1994; 310(1): 32–39. DOI: 10.1006/abbi.1994.1136
- Patuzzi RB. A model of the generation of the cochlear microphonic with nonlinear hair cell transduction and nonlinear basilar membrane mechanics. *Hear Res*. 1987; 30(1):73–82. DOI: 10.1016/0378-5955(87)90185-7 [PubMed: 3680056]
- Patuzzi RB, Yates GK, Johnstone BM. The origin of the low-frequency microphonic in the first cochlear turn of Guinea-pig. *Hear Res*. 1989; 39(1–2):177–188. DOI: 10.1016/0378-5955(89)90089-0 [PubMed: 2737964]
- Pierre SV, Sottejeau Y, Gourbeau JM, Sánchez G, Shidyak A, Blanco G. Isoform specificity of Na-K-ATPase-mediated ouabain signaling. *Am J Physiol Ren Physiol*. 2008; 294(4):F859–F866. DOI: 10.1152/ajprenal.00089.2007
- Schmiedt RA, Okamura HO, Lang H, Schulte BA. Ouabain application to the round window of the gerbil cochlea: a model of auditory neuropathy and apoptosis. *J Assoc Res Otolaryngol*. 2001; 3(3):223–233. DOI: 10.1007/s1016200220017 [PubMed: 12382099]
- Schmiedt RA, Zwislocki JJ. Low-frequency neural and cochlear-microphonic tuning curves in the gerbil. *J Acoust Soc Am*. 1978; 64(2):502–507. [PubMed: 712012]
- Suzuka Y, Schuknecht HF. Retrograde cochlear neuronal degeneration in human subjects. *Acta Otolaryngologica Suppl*. 1988; 450:1–20. DOI: 10.3109/00016488809098973
- Whitfield IC, Ross HF. Cochlear-microphonic and summating potentials and the outputs of individual hair-cell generators. *J Acoust Soc Am*. 1965; 38:126–131. [PubMed: 14347603]
- Withnell RH. Brief report: the cochlear microphonic as an indication of outer hair cell function. *Ear Hear*. 2001; 22(1):75–77. [PubMed: 11271978]

Fig. 1.

(a) Schematic portrayal of typical CAF curve (solid line) and CAF curve post damage to apex (dotted line). (b) Schematic portrayal of hypothesized CAF curve with steeper growth in apical responses (solid line) which would more potentially show a more dramatic change when damaged (dotted line).

Fig. 2. CAP amplitudes after application of ouabain or AP-only (control) as a function of signal level and paneled by stimulus frequency. Solid lines are group means and shaded regions represent 95% confidence intervals.

Fig. 3. DPOAE amplitudes after application of ouabain or AP-only (control) as a function of signal level and paneled by stimulus frequency. Solid lines are group means and shaded regions represent 95% confidence intervals.

Fig. 4. CAF curves for 45 Hz (top), 85 Hz (middle), and 762 Hz (bottom) at 80 dB SPL (left) and 90 dB SPL (right). Colored diamonds indicate individual animals' data and black lines show the fitted regression lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Unmasked CR amplitudes for 45 & 85 Hz at 80 & 90 dB SPL. Each panel shows the mean pre-treatment (black) and post-treatment (gray) amplitudes for the ouabain and AP-only (control) groups with standard error bars. ANOVA on the differences between pre- and post-treatment show significant differences (*) between control and ouabain groups in all conditions except for 45 Hz, 80 dB SPL.

Fig. 6. Differences between pre- and post-treatment CR amplitudes in filtered noise conditions for ouabain and AP-only (control). Solid lines represent group mean difference scores and shaded regions are 95% confidence intervals.