University Doctorate (Ph.D.) Essay Thesis

PERIPHERAL FINE MODULATION OF HEARING: SLOW MOTILITY AND ELECTROMOTILITY OF OUTER HAIR CELLS

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Introduction

The mammalian cochlea is an especially sensitive hydro-mechanical frequency analyzer, which has the function of sensing and processing sound's frequency and intensity in real time in form of action potential in the auditory nerve. It has the capability of sensing both very low and very high intensity sounds at a very wide spectrum of frequencies. Further more in certain species (such as bats) it has the capability of differentiating consecutive sounds at intervals of ~10 μsec at 100 000 Hz. A complex mechanical system is set up due to its built. Based on the theory of travelling waves of Békésy, the passive mechanical characteristics of the basilar membrane, enables it to analyze frequencies of sounds. The ossicular chain movements caused by the sound waves, is conducted to the perilymphe fluid compartment, which brings the cochlea to motion: basilar membrane - organ of Corti - tectorial membrane unit (cochlear sound perceiving system). Now we know that the travelling wave theory of Békésy applies only to non-living "passive" cochlea, and only intense stimuli can provoke it, where its frequency resolution is considerably rough. Nevertheless, all this is not enough for cochlea's true sound perceiving dynamic range and its frequency tuning. In the "active" cochlea, on the basilar membrane the sound waves travel in a similar fashion as the travelling wave with the difference of both quality and quantity. The tuning is sharper and certainly non-linear. The cochlea's attributes are due to its unique structure of mechanically active cells that is the outer hair cells function.

The mammalian hearing organ and its micromechanical chracteristics

On the cross section of the bone cochlea one can find the triangular membranous labyrinth. This divides the boney canal into scala vestibuli and scala tympani, which are located between the bone and membranous labyrinth. A third compartment is located within the membranous labyrinth and it is called cochlear duct. The cochlear duct is separated from the scala vestibuli by the Reissner's membrane. Its lateral wall is formed by stria vascularis, and it is separated from the scala tympani by the basilar membrane. All three compartments are filled with fluid. The cochlear duct is filled by endolymph, whereas scala tympani and scala vestibuli is partly filled by plasma ultrafiltrate and partly by perilymph derived from cerebrospinal liquor.

The organ of Corti is a spiral cellular matrix located on the basilar membrane facing the cochlear duct. The receptor cells of the organ of Corti are called hair cells. Two types of such

cells are differentiated, outer and inner hair cells. On the two sides of the Corti tunnel, in the space between the outer pillar cells and Hensen cells, resting on synaptic poles on Deiter's cells, with its ciliated poles joint to the tectorial membrane, in 3-4 rows are the outer hair cells located. Next to the inner pillar cells, surrounded by supporting cells, the inner hair cells are found in one row. Approximately, 3500 inner hair cell are found in cochlea, from the base to the apex. Since the basilar membrane is permeable to ions, the Corti organ is literally bathing in perilymph that fills the scala tympani.

The cellular architecture of the organ of Corti is determined by the organization of the supporting cells. The lateral membrane of the outer hair cells located next to the Deiter's cells is in direct contact with the perilymph filling the Corti tunnel. The hair cells cuticular surface on the top of the pillar cells, the Deiter's cells and other supporting cells apical membrane towards the endolymphatic space make a closing disc with the *zonula occludens*. This forms the lamina reticularis which is a network-like frame. The space between the tectorial membrane and lamina reticularis is devoid of cells and contains collagen. Ductus cochlearis filled with endolymph has a free passage in this space. The lamina reticularis and the apical surface of the hair cells with its stereocilia are incubated in the endolymph.

Structure of the outer hair cells

The outer and the inner hair cells have structural differences. The common thing between the two is that, from their apical membrane the so called stereocilia is projected. These projections contain actin filaments and are covered by plasma membrane. From the base towards the apical region these projections become fewer and thinner. In the inner hair cells projections are in one line, whereas in outer hair cells they are grouped in forms of "W", "V" or "U". Cross bindings are found between the stereocilias, that cause a high degree of stiffness as in a bouquet. This enables the bouquet to move as a unit in case of movements applied to the longest stereocilia. The longest stereocilias are in contcat with the lower surface of the tectorial membrane. In a similar fashion there are connections like a framework between the hair cells that have their stereocilia connected. The tip of the shorter stereocilias are connected to the sides of the longer stereocilias. These are called "tip links", which has mechano-sensitive transduction channels on the endings of the shorter stereocilias.

Since morphologically the stereocilias are in contact with the tectorial membrane, this cause the stereocilias of the outer hair cells to bend towards the stria vascularis simultaneously as the tectorial membrane moves towards the scala vestibuli. The bending of the stereociliary bundle in the direction of its longest stereocilias causes tension in the tip links, which is the inducing stimulus for transduction of the channels to open. Synchronous to the displacement, the cation channels on the tip of the stereocilias opens leading to the influx of K^+ from the endolymph which has a high concentration of K^+ into the cells, causing depolarization of cell membrane.

The lateral wall of the outer hair cells is special in a manner that it is made of three lengthwise concentric cylinder layer, with thickness of ~100 nm. The outer layer is the plasma membrane that differs from the one found on the lateral wall and the apical pole region. Among the differences, the lateral wall plasma membrane is a modified anion exchanger, glucose transporter, and contains a protein called perstin which forms the basis for electromotility of the outer hair cells.

The lateral wall's inner layer is the membranous subsurface cistern. The membranous part is in continuation with the membrane of the canalicularis reticulum found in the apical pole. The structure of the subsurface cisterns is similar to the structure of the endoplasmic reticulum. The mitochondrions of the hair cells are mainly near the subsurface cistern. Continuity is seen between the outer membrane of mitochondrions and the membrane of the subsurface cisterns.

The so called cortical lattice is found between the two layers. In its structure two particular type of protein can be found (F-actin and spectrin) which can also be found in cuticular lamina. The F-actin and spectrin in the cuticular lamina are arrayed in a vertical manner and can be found in the entire length of the lateral wall and in continuation with the cortical lattice. Among the circularly arrayed F-actin filaments distanced at ~40 nm, the spectrin filaments are stretched in longitudinal arrays. Pillars radial to the plasma membrane fix the F-actin bundles running parallel and their neighboring subsurface cisterns in a lasso-like manner. The parallel running F-actin bundles create a special structural unit like a "microdomain" that is capable of changing its shape in response to changes of membrane potentials and also the capability for storage of flexible energy.

Function of the outer hair cells

The mammalians outer hair cells are mechanically active. These sensori-motor cells regulate the character of response induced by sound stimulus to the organ of Corti. They are also responsible for cochlear amplification of sounds at near threshold. The travelling wave at around auditory threshold is actively amplified by the outer hair cells, so it will have enough intensity required to excite the receptors of the inner hair cells. This is known as the cochlear amplification. This phenomenon is non-linear and it loses its importance at threshold values of above 50 dB.

The outer hair cells have two motor activities, conformingly with the cells long axis. The slow (accomplished in seconds to minutes) motile cell response (shortening-relaxation) and the fast or electromotility (in μsec to msec).

The inner hair cells recieve almost exclusively afferent, whereas the outer hair cells recieve definitively efferent innervations.

The cholinergic neurotransmission results in influx of Ca^{2+} which creates Ca^{2+} freeing induction. The Ca^{2+} signal starts different processes of cytoskeletal unit phosphorylation, which enables the outer hair cells changing its mechanical characteristics. In case of GABA similar to ACh effect inducing dose dependent reversible hyperpolarization of the membrane is noticeable.
The phosphorylation of the cytoskeletal proteins changes the stiffness of the lateral wall and through this the outer hair cells active energy feedback, or in other terms the cochlear amplification.

The mechanically active outer hair cells' slow motility causes cell shortening in a 20-60 seconds time interval. The slow structural motility change of the outer hair cells resulting in shortening, is an inducible mechanical (fluid flow) or with chemical effect (K^+) by depolarization, with electric gradient, and osmotic effect or rise in intracellular Ca^{2+} level.

The slow motile cell response requires ATP. The cytoskeletal reorganizations managed by means of phosphorylation and dephosphorylation of cytoskeletal proteins such as actin and spectrin. A physiological role of the slow motility in the protection of the cochlea is cochlear adaptation.

The sinusoid sound waves elicite electromotility by means of the receptor potential in real time leading to changes in the cells length (shortening and lengthening), which follows the frequency and is coupled with phase (up to 5000 Hz). The electromechanical transduction causes

fast electrokinetic cellular changes. This is capable of shortening and lengthening the cell in nanometer magnitude compared to resting audition. Depolarization causes shortening, hyperpolarization causes elongation. Electromotility of the outer hair cells followes the frequency of the sound. Voltage-dependent movements of the outer hair cells, caused by sound-vibrated basilar membrane, has a physiological role. The role is that it feeds energy back to the basilar membrane.

At around thereshold sound intensity the fast electromotility provides with the basis for cochlear amplifier. The electromotile shortening and elongation of the outer hair cells which follow cycle-by-cycle the frequency of the sound are produced by a concentrated action of the voltage-dependent conformational changes of the prestin molecule, the motor protein.

The prestin is a polypeptide of 744 amino acids. It has 12 transmembrane domains. On the effect of voltage pulse it binds chloride ions and moves them toward the extracellular side of the cell membrane, but does not transport it (incomplete transport). Membrane potential change, simultaneously causes anion movement and changes in the conformation of protein. The conformation changes in prestin molecule are exclusively driven by membrane potential changes, which are independent of intracellular ATP. The conformational changes are summated at the cellular level and the cell shape change-driven force is dependent on cytoskeletal status.

Prestin based electromotility in conjunction with cell membrane and cytoskeleton forms functional units for the cell shape changes (microdomains). These structural units and their actual condition, determin the outer hair cells stiffness. The prestin molecules apply force against cell stiffness. Cell stiffness is the function of the cytoskeletal geometry and regulated by metabolic processes (e.g. phosphorylation).

The outer hair cells' motility is regulated by different metabolic pathways. Among these the highly regulated Ca^{2+} signaling plays a significant role in the outer hair cells. The intracellular Ca^{2+}concentration has a role in the efferent neurotransmission (ACh), and in the regulation of the cGMP dependent kinases and the Rho-kinases (Rac1, RhoA, Cdc42).

Otoacustic emission is the only clinically accepted audiological test which proves the mechanical activity of the mammalians' cochlea. Otoacustic emission is a retrodrade propagation of vibrations from inner ear toward the middle ear which are produced by electromotile oscillations of the outer hair cells.

The outer hair cells have a uniquely structured lateral wall that we have discussed earlier. This special structure leads to two special mechanical characteristics. It influences the cell wall

stiffness, which is prestin (motor molecule) independent, and it enables prestin to regulate the outer hair cells function mechanically providing with electrokinetic shape change.

Lateral wall stiffness change of the outer hair cells influences the affectiveness of the electromotility. In this process, presumably the most important role is played by the cytoplasmic surface of the plasma membrane and the subsurface cisterns that is tightly connected to the cortical lattice. The cytoskeleton maintains the resilient pre-stressed state. This ensures the hair cells' resting mechanical resistant so they maintain their cylindrical shape. The effectiveness of outer hair cells' motor activity and consequently the efficacy of the cochlear amplifier longitudinal and circumferential stiffness and global cell stiffness are primarily influenced by the lateral walls stiffness.

The slow motile cell response changes the geometry of organ of Corti and the cochlear micromechanics, which modifies the cochlear amplification. Presumably, this serves as a protective mechanism against harmful effect of too laud sounds. The reversible slow motile cell response and the resulted changes in lateral wall stiffness is not yet understood well.

Objectives

1. Examination of slow motile outer hair cell response to mechanical (noise model) and chemical (K^+ intoxication: Menière disease) stimuli.
2. Studying the effect of outer hair cells slow motility on the lateral wall stiffness.
3. Studying the influence of slow motile length change upon magnitude of electromotility.
4. Studying the role of phosphorylation in the regulation of outer hair cells lateral wall stiffness.
5. Studying the relationship between the magnitude of reversible slow motile response, the lateral wall stiffness and the magnitude of electromotility of the outer hair cells.

Materials and Methods

Cell isolation

Either sex of 3-5 weeks old, pigmented guinea-pigs with positive Preyer's reflex were euthanized by intraperitoneal pentobarbital injection. The bulla osseae were excised and the lateral wall of the cochleae and the stria vascularis were removed. The turns of the organ of Corti were dissected and the outer hair cells were isolated enzymatically at room temperature with collagenase (type IV Sigma, 1 mg/ml). Pieces of the organ of Corti were transferred to an experimental chamber filled with 400 µl of Hank's solution. Outer hair cells were dissociated by gentle mechanical trituration. The cells have an average length of 74 ±4 µm (n=92). Extracellular solution contained (in mM): 136,75 NaCl, 5,36 KCl, 0,44 KH_2PO_4, 0,34 Na_2HPO_4, 1,26 $CaCl_2$, 0,81 $MgSO_4$, 5,56 glükóz, 4,17 $NaHCO_3$, 10 HEPES (300 mOsm/l, pH 7,4).

The cells showed signs of life through their physiological function, but in general the following criteria were considered for the cell to be eligible for the experiments:

1. Normal positioning of the cell nucleus (basal)
2. Tense, elongated cell shape (good turgor)
3. Intact stereociliary bundles under light microscope.
4. Absence of Brown movements in the cytoplasm.

The cells on average are capable of maintaining these criteria for two hours.

Microscopic study of the outer hair cells, measurements of the two types of motile activity and lateral wall stiffness.

We performed our measurements on the isolated outer hair cells in an experimental chamber (400 µl) filled with Hank's solution, using a Zeiss Axiovert 100 inverted phase contrast microscope, with 400x magnification. The entire measuring system was placed on a vibration free table (Newport VH 3660W-OPT) and it was surrounded by a Faraday cage. The motile cell response was detected on an oscilloscope, and the images were digitized. For measuring the lateral wall stiffness we digitally recorded the phase contrast images, and for better visibility we performed a pseudo-color conversion.

Used technical instruments and programs

Length changes (slow and fast motility) of the OHCs were mesured by photocurrent change of a photodiode (PIN-10D), when magnified images of the ciliated poles of the cells were projected onto it through a rectangular slit. Photocurrent responses were amplified, low-pass-filtered (6 dB cut-off, frequency of 1 kHz), detected by an oscilloscope (TDS 220, Tectronix) and digitized. For the purpose of digitalization of the photo current changes on a computer we used the software provided by Tectronix (WaveStar for Oscilloscopes, version 2.6).

We applied the Farkas and Szikali (2003) micro-flow method for induction of slow motile cell response.

For the cell's electromotility measurements the voltage command stimuli were a series of rectangular pulses of alternating polarity (hyperpolarization and depolarization), increasing in magnitude in 7 steps, were generated by a programmable computer driven system (IBM AT clone).

We studied the stiffness of the outer hair cells lateral wall by aspiration micro-deformation technique. The phase contrast images of magnified cells were recorded by Sony DXC-107P video camera connected to a computer with external digitalizing card (Pinnacle Systems, INC. USA, California). Off-line after digital cleaning and noise freeing, using a custom designed software we performed pseudo-color alteration.

For the purpose of digitalization and data analysis we used a computer with 2.6 GHz Intel Celeron processor, 256 MB RAM, ATI Mobility Radeon 9600 video card and 30GB IDE HDD configuration.

Measurement of the outer hair cells slow motile response

The experimental bath, which contained the isolated outer hair cells, was placed on the stage of the microscope. The outer hair cells were inserted into a glass microchamber with an inner diameter of ~9 μm by the synaptic pole first using negative pressure. The microchamber used, were made of Clark EC 15 TF glass in a two step forming process. We smoothened it by applying heat, and made the tip in a way that the inner diameter of the very end of it just fits the outer diameter of a hair cell, while the rest of the glass suddenly expands. The microchamber and

the inserted cell (approx. 10% of the cell length, e.g. 6-8 µm) segment formed a mechanically stable seal, but the cell could be moved in and out of the pipette.

The outer hair cells' motile response (shortening, relaxation) was measured in the following way: the magnified image of the cell's ciliated pole fixed in the microchamber was projected through a narrow rectangular slit on to the PIN 10D photodiode, and the photocurrent change caused by movements was measured. The cells longitudinal small changes (in µm magnitude) change the darkened part of the slit, and the photo current coming from photodiode is modulated. The cell was monitored by a camera while being suctioned in the microchamber and also during the measurement process. We also monitored the cells position in the slit (2 video camera cell monitoring).

Photocurrent responses of length changes were calibrated manually before experiments by a stepper motor rotated optical lever (1.8% step, equals to changes above ~200 nm). Amplified, low-pass-filtered, and detected by an oscilloscope. The photocurrent was digitalized using a computer.

We measured the motile response of the parts of the hair cells falling out of the micro-chamber. For induction of slow motile cell response we used the Farkas and Sziklai (2003) method of micro-flow. We made glass capillary from Clark EC 15TF perfusion pipettes with a 2 step forming program with inner diameter of ~50 µm. We attach the pipettes to a plastic tube through a polyethylene tap to an open plastic tank with capacity of 2 cm^3. We equalized the fluid level needed for the measurements of 400 µl to the fluid level of the experimental chamber, so the fluid in the perfusion pipettes wont flow in neither outwards nor inwards direction. The perfusion pipettes opening is placed ~150 µm from the cell, in a way the openings lower edge is resting upon the fluid chambers button, parallel to the cells longitudinal axis as continuum.

We induced slow cell motile response with extracellular fluid flow at speed of 0.6 µl/min (Hank's solution) (n=9) (mechanical stimulation), or 12.5 mM KCl solution (n=9) (mechanical and chemical stimulation). From our previous experiments, we found out that, the magnitude of the motile response is proportional to the perfusion speed. The reason for choosing this flow speed is that during our previous experiments the magnitude of induced motile cell response (< 1.5 µm) remained in physiological range. With both type of fluid flow inductions, non-selective serine/threonine protein phosphatase inhibitor in the presence of ocadaic acid (0.6 µM/l) and absence of it (n=9 in both experiments), the duration was 90 seconds. Prior to beginning the experiments, for the purpose of treating the outer hair cells with ocadaic acid we incubated it for

30 minutes. We continuously registered the slow motile cell response for 225 seconds. After starting the experiment, we began the fluid flow in 45 seconds, which lasted 90 seconds. A 90 seconds period of relaxing time (expansion) of no flow was followed.

Measurement of the outer hair cells electromotility

We performed measurements of electromotility induced on outer hair cells by electric square pulses. We equally measured the magnitude of electromotile responses of mechanical (external solution flow) and chemical stimuli (12.5 mM KCl solution perfusion) in non-selective serine/threonine protein phosphatase inhibitor in the presence of ocadaic acid (0.6 μM/l) and absence of it (n=9 in both experiments).

In all experiments before starting the flow (20 seconds), during the slow motile cell response period caused by perfusion (shortening) (60-120 seconds), and in recovery period (expanding) (150-170 seconds), the measurements were preformed at the following intervals: 20, 60, 80, 120, 150 and 170 seconds.

We placed the chamber filled with extracellular solution containing isolated outer hair cells under the microscope, and then we applied the experimental protocol used for measuring the slow motile cell response. The electromotility measurements were performed during the slow motility process in the above mentioned time intervals. The voltage command stimuli, a series of rectangular pulses of alternating polarity, increasing in magnitude from ±35 to ±240 mV in 7 steps, were generated by a programmable computer driven system. Each pulse was 24 msec long with 60 msec resting period between each two pulses.

Transcellular electric stimulation was created between the incubating solution both inside and outside of the pipette. The stimulating voltage is ensured by an electric voltage converter. A computer controlled the wave shape, synchronization and intensity.

We measured the motile response of the outer hair cell parts falling out of the microchamber. Before starting each stimulus series, we calibrated the photocurrent as described earlier.

From the changes in the magnitude of electromotility responses we can conclude the scale of cochlear amplifier in these ways: 1- reduction of magnitude of motile response corresponds to in vivo perceptional hearing loss, or protective automatization, 2- increase in the magnitude of motile response is perhaps responsible for the mechanism of fine physiological tuning of threshold sensing.

Study of the outer hair cells lateral wall stiffness

Stiffness of the lateral wall was mesured by the glass micropipette aspiration technique. In this process we measure the cell wall's deformation against pulling force. We applied known negative pressure (4-10 H_2O cm which keeps the cell walls 3 structural layer still intact) with micropipette on the cell's lateral wall. We determine the lateral wall stiffness by the amount of retraction in the pipette. Determination of micro-deformation is done by visual analysis of the outer hair cell. For acceptable accurate measurements we need enough magnification, enough resolution and good cell boundary contrast from the background.

Glass micropipettes were pulled from Clark EC15TF capillaries in five stages, the inner diameter was 2.8 ±0.2 µm. Pipettes were filled with Hank's solution and connected to pressure application apparatus by a polyethylene tube. The later wall retraction was measured in the presence of 6 cm H_2O aspiration pressure. We performed the measurements in a plastic experimental chamber filled with 400 µl of physiological extracellular fluid, under a phase contrast microscope with 400 X magnification. The slow motile cell response was induced by mechanical and chemical stimuli, in the presence and absence of ocadaic acid ((0.6 µM/l) and at a specified time we adjusted the micropipette to the outer hair cells lateral wall and applied the negative pressure, then after 30 seconds the amount of retracted cell wall was measured. The aspirated lateral wall segments were magnified by phase contrast microscope, videorecorded (CCD video camera), digitized (with an external digitizing card) and analysed off-line after pseudocoloration

A custom developed program in the Turbo Pascal language was used for pseudocoloration. After digitalizing the microscopic photo, cell particles with different optical densities were depicted as different pixels in a grey scale. After adjusting the obtained digital photo bitmap (bmp) in pixel information matrix format the result became suitable for different algorithmic computer processing. The pixel information configured by the selected algorithm is assigned to a standard VGA color scale. Pseudo-color images were also obtained using contrast enhancement and digital image subtraction. The resolution of harvested contrast photos obtained this way is 18 pixel/µm. After this the value of the later wall stiffness (S_p) was determined based on the below equation:

$$S_p = \frac{-\Delta P \cdot r^2 \cdot \pi}{\Delta L} \qquad (1)$$

Where ΔP (nN / μm^2) is the applied negative pressure in the pipette, r (μm) is the micro-pipettes inner diameter, ΔL (μm) is the part of the lateral wall retracted into the pipette.

Mathematical methods, statistical analysis

For calculating the outer hair cells' lateral wall stiffness, we used the Euler model (equation 1).

We applied a modified hit and miss model to describe the outer hair cell stiffness dependent electromotility changes. In this process we assumed that the outer hair cells electromotility is made up of two parts: one that is controlled by the later wall stiffness, and one part that is not controlled by the later wall stiffness. In our electromotility model, the lateral wall stiffness controlling effect fulfills in a way that increases in the lateral wall stiffness seems to causes the electromotile units to become kinetically inactive. In our model the inactivity refers to the created functional inactivity secondary to increase in the lateral wall stiffness and not to the destruction of the units of electromotile structure leading to functional loss (equation 2).

To study the significance of noticed bias during our experiments we used the Student type double t-assay probe. SD-t means the given coefficient of standard deviation.

Results

The outer hair cells slow motile response to mechanical and chemical stimuli

Slow motile shortenings of isolated outer hair cells can be evoked by mechanical or chemical stimulations. Mechanical stimulus, a low-rate (0.6 μl/min) perfusion of saline into the incubation chamber, resulted in a reversible outer hair cells slow shortening. The maximal cell shortening was reached at ~75 seconds after the start of the flow (774 ± 87 nm) (n = 9) and did not exhibit any further cell contractions.

The magnitude of slow shortenings evoked by perfusion of 12.5 mM KCl solution at the same flow-rate was faster and larger (1465±159 nm) (n=9). The maximal slow motile cell response in this case was also ~75 seconds after the begining of the flow, which did not increase by the end

of the fluid flow. Cell length recovery to their near original sizes was obtained after both type of stimulations.

Slow motile cell response wasn't inducible neither with mechanical stimulus, nor by a combination of mechanical and chemical stimuli after preincubation of the outer hair cells for 30 minutes in a non-selective serine/threonine protein phosphatase inhibitor with ocadaic acid (0.6 µM/l). The resting cell length remained unchanged during the flow until the end.

The outer hair cells slow motility and the lateral wall stiffness

Lateral wall stiffness of the outer hair cells at different stages of the slow motile cell response is measured at the above mentioned time intervals. Control value of lateral wall stiffness was 1.25±0.03 nN/µm. Mechanical stimulation by perfusion of extracellular solution increased lateral wall stiffness continuously (1.39 ±0.03 nN/µm) but to a lesser extent than perfusion of 12.5 mM KCl (1.52 ±0,03 nN/µm). Return to baseline stiffness value was obtained in both stimulation protocol after 35 s resting time (at the timepoint of the experiments of 170 second). The magnitude of lateral wall stiffness-increase seems to be dependent on the degree of slow motile shortening irrespective to which stimulation - mechanical or chemical - elicits the cell shape changes. Ocadaic acid increased the baseline (no perfusion) stiffness of the lateral wall after 30 min incubation time (1.52 ±0,04 nN/µm) without changing the resting cell length. Mechanical or chemical stimulation, however, can not elicite any further change in stiffness of outer hair cells preincubated by ocadaic acid.

The outer hair cells slow motility related changes in electromotility

Electromotility measurements at different stages of slow motile shortenings were achieved at 6 time points, during experiments: before electromotile activity was elicited, during slow motile shortening and after a recovery period following the mechanical or mechanical + chemical stimuli. Irrespective to the stimulus - mechanical or mechanical + chemical - the magnitude of fast motility depends on the slow shortening induced lateral wall stiffness change. As greater is the outer hair cell shortening elicited lateral wall stiffness increase that smaller is the magnitude of the fast motility magnitude response both to lowest voltage (±35 mV) and maximum voltage electrical stimuli (±240 mV). This note stresses the probability of a systemic effect of the cell-

stiffness increase upon the hair cell motor function, since the smallest applied voltage step is designed to be mimicking the near-threshold receptor potential whereas the maximum applied voltage step is far in the saturation range of electromotile activity. Relation of the magnitude of fast motility to length of the outer hair cell is independent from whether it was measured during the shortening or in the recovery period of the cells. The characteristics of the electromotile responce curve to lowest voltages and maximum applied voltages are similar, but low woltage-response shows saturation kinetics.

Effect of ocadaic acid on the outer hair cells motility and lateral wall stiffness

In the presence of ocadaic acid, a nonspecific serine/threonine phosphatase inhibitor, neither mechanical nor chemical stimulation can evoke slow motile response of the outer hair cells. Increase in the lateral wall stiffness and decrease in the magnitude of fast motility is observed, however. Ocadaic acid arrested the responsiveness of outer hair cells slow motility, lateral wall stiffness and electromotility to mechanical or chemical stimulation

Discussion

Electromotile force feedback by the outer hair cells is a key mechanism in the sensitivity of sound perception at low intensity signals in mammals. Present experiments examine 3 consutive cellular response characteristics of the outer hair cells to better understand those influences and mechanisms which may regulate the efficacy of the cochlear amplifier (outer hair cells electromotility) in physiological and pathological conditions. These response properties of the isolated outer hair cells are (i) evoked slow motile shortening, (ii) related increase of the lateral wall stiffness (iii) and corresponding decrease in the magnitude of electromotile performance of outer hair cells.

Slow motile shortening of isolated outer hair cells could be induced by both mechanical and chemical challenges. This cell length decrease can exceed 1.5 μm (2% cell length) in outer hair cells. This should evoke a considerable dystortion of the cellullar matrix in the organ of Corti which can change its micromechanical responsiveness to sound stimulation. Important to note

how small mechanical stimulus can already elicite the slow cell shortening. A perfusion rate of saline of 0.6 µl/min (10 nl/s) flow is comparable to those fluid pressure changes which develops upon the basilar membrane due to the traveling wave: This perfusion induces a 5±0.8 µm/s flow rate on the cell surface facing towards the perfusion pipette and a 2.8±0.22 µm/s flow rate on the opposite cell side as determined by measurement of the velocity of traveling particles under the microscope. Taking the Bernoulli law,

$$\Delta P = ½ \rho (v_1^2 - v_2^2),$$

where ΔP is the pressure which is applied to the cell surface on the side facing towards the perfusion pipette, ρ is density, v_1 and v_2 are flow rates measured on the two opposing sides of the cell, the pressure applied to the cell surface during the experiments (ΔP) were 8.4×10^{-9} N/m^2. This value is in the same order of magnitude of those pressure changes which is generated by the velocity difference of the traveling wave in the distance of the diameter of one outer hair cell ($\Delta P = 2.46 \cdot 10^{-9}$ N/m^2).

Development of slow shortening of outer hair cells to small mechanical stimuli is probably dependent on opening of stretch receptors and movement of the stereociliary bundle. In our preparation, the cell isolation technique *ab ovo* distroys the tectorial end of the stereociliary bundles and the tiplinks. Movement of the free-standing stereocilia of the isolated outer hair cells due to the mechanical insult produced by the perfusion opens mechanosensitive channels and allows a cation influx. Both stretch receptors in the lateral plasma membrane and mechanoreceptors in the stereocilia operate with calcium. Elevated intracellular calcium concentration, $[Ca^{2+}]_i$, results in a metabolic change (phosphorylation/dephosphorylation) in the actin and associated cytoskeletal protein network of the cortical lattice. The concomitant change in the assembly of the actin-spectrin network leads to shortening of outer hair cells.

Magnitude of mechanically induced slow outer hair cells shortening is further increased by elevation of the K^+ concentration in the incubation fluid to 12.5 mM. Both mechanical and chemical stimulation-induced slow outer hair cells shortening can be arrested by addition of ocadaic acid into the bath. The influence of K^+ on outer hair cells in doubling the mechanically evoked slow shortening can only partially be explained by elevation of $[Ca^{2+}]_i$ as in calcium-free extracellular solution the cells still can perform slow shortening. This argues for several signaling pathways involved in the development of slow motile shortening of the outer hair cells. One is

calcium-dependent and another, which is inhibited by ocadaic acid, a protein phoshatase inhibitor, is phosphorylation/dephosphorylation-dependent.

Evoked, reversible, slow shortening of outer hair cells results in a stiffness increase of their lateral cell wall. Lateral wall stiffness of 1.25 nN/μm in our experiments is in the range (0.8 and 3.3 nN/ μm) of those measured by others. The stiffness increase is independent from the type of the stimulus as to mechanical or chemical. The greater the cell shortening the more pronounced the lateral wall stiffness increase. Approximately 2 times greater cell shortening is due to mechanical+chemical challenge than to mechanical stimulus alone. Simultaneously, the lateral wall stiffness increases linearly. K^+ induced (mechanical+chemical stimulation) stiffness increase is double of that of the perfusion (mechanical stimulation) induced stiffness increase.

Electromotility magnitude follows inversely the lateral wall stiffness increase**.** Considering the fact that the outer hair cells are included in a microchamber and the microchamber is a voltage devider, the magnitude of the electric pulse and the extrusion factor[1] (q=9) considering that stimulus potential is 1/10 of square pulse. In the case of smallest stimulus (±35mV) this is 3.5 mV, which is a value of similar magnitude to the receptor potential of near threshold intensity sounds. With the maximally ±240mV applied square pulse intensity (24 mV on the cell membrane) the magnitude of electromotility is in the saturation range. These values of membrane voltage changes caused by receptor flow fell in physiologic range.

The increase in the mechanical load upon the prestin molecules due to stiffness increase, decreases the magnitude of the electromotile responses. Again, no difference was found in electromotility magnitude decrease caused by mechanical or mechanical+chemical stimulation-induced lateral wall stiffness-increase indicating that the underlying molecular mechanism in the development of outer hair cells shortening is common or synergistic mechanisms (1 mechanically and 1 chemically induced) work together. Lowest voltage (±35 mV) electromotility response magnitude obtaines a saturation by mechanical stimulation alone in the function of both slow shortening and lateral wall stiffness increase. The electromotility response magnitude, and hence the cochlear amplifier, is dependent on the lateral wall stiffness which can be due to slow motile shortening. This regulatory mechanism may provide with setting the force feedback of the cochlear amplifier to an appropriately low level which is about 1/3rd of control values. One component in the regulation of electromotility magnitude is cell stiffness-dependent change in

[1] Expresse the amount of the outrer hair cell being retracted in the micro-chamber: q=0.9 which means that 10% of the cells are inside the micro-chamber.

electromotility. Another factor derives from stiffness increase to a level where the conformational change of prestin can not occur or the signals are too small to be detected by the available techniques. Hence, this can be described by a modified target theory model. Continuous quasi-inactivation of prestin molecules can be the consequence of stiffness differences in the lateral wall of outer hair cells along the cell axis as well as of slow shortening induced different stiffness changes in different cell segments. The prestin molecule's continuous "inactivity" is secondary to the increase in the inhomogeneous stiffness caused by the slow motile response. The "inactivity" does not mean that the prestin molecules are indeed inactive, they only work against a larger mechanical load. It is also possible that the prestin is functional but the summation at cellular level is failed.

The model is as follow:

$$y = y_0 + a \cdot e^{-bx} - c \cdot x \qquad (2)$$

where y is magnitude of electromotility, y_0 is the non-regulated magnitude of electromotility, a is the basic, regulated magnitude of electromotility, b is quasi inactivation coefficient, c is the stiffness-dependency constant of the electromotility, x is the stifness of the outer hair cell lateral wall, e is the natural number.

Lateral wall stiffness can be increased in the absence of slow shortening, too, resulting in a fast motile performance decrease similar to stiffness increase due to slow shortening. This phenomenon was observed when outer hair cells were preincubated in ocadaic acid.

Ocadaic acid inhibits both protein serine/threonine phosphatase 1 (PP1) and protein serine/threonine phosphatase 2A (PP2A) in the concentration applied (0.6 μM). Its capability to block phosphatases of tyrosine phosphorylated proteins is negligible. Consequently it seems, that serine/threonine phosphorylated proteins play a role in outer hair cells as part of the cell shortening process.

A calcium-dependent phosphorylation was also considered in the process of the acetylcholine (ACh) evoked electromotility magnitude-increase. Whereas, however, slow motility induces lateral wall stiffness increase, acetylcholine causes stiffness decrease. Present findings, that ocadaic acid blocks the development of slow motility, increases lateral wall stiffnes and concomitantly decreases the magnitude of electromotility by elevating the phosphorylated forms

of target proteins are seemingly opposite to what one expects since ACh was assumed to cause stiffness decrease also through phosphorylation of probably the same cytoskeletal molecules.

A possible explanation for phosphorylation induced lateral wall stiffness increase or decrease can be the diversity of regulatory cascades in activation of protein kinases and phosphatases. Ocadaic acid does not block dephosphorylation of RhoA by PP1 at the present concentration. Phosphorylated RhoA does not inhibit Rac-1 which subsequently decreases the magnitude of electromotility, shortens the outer hair cells and probably increases lateral wall stiffness. This electromotility magnitude decrease was measured to be around 40% which is very similar to those what we have measured after addition of ocadaic acid. ACh, on the contrary, sets outer hair cells length from shortened shape to resting length, decreases stiffness and increases the magnitude of electromotility. Protein phosphorylation can determine the degree of shortening of the actin-spektrin network in the cortical cytoskeleton resulting in stiffness increase/decrease on a given scale.

It is reasonable to assume that phosphorylation of target cytoskeletal proteins results in a stiffness decrease without outer hair cells shortening whereas activation of Rho kinases (phosphorylation) through Rac-1 results in a stiffness increase.

Mechanosensitvity of outer hair cells in eliciting tonic length changes is considerably high and presents an important local feedback regulation in sound perception and to protect the organ of Corti from loud sounds. Outer hair cells shortening decreases the electromotility feedback through a cell stiffness increase. This is a safety feature of the auditory periphery which can set the actual gain of the cochlear amplifier and can, simultaneously, serve as an adaptation procedure against the noisy environment.

The thesis underlying *in extenso* publications

Contributions

1. Borkó R., Batta J.T., Sziklai I. Electromotile performance of isolated outer hair cells during slow motile shortening. Acta Otolaryngol 125(5), 547-551, 2005. [IF.: 0,79]
2. Borkó R., Batta J.T., Sziklai I. Slow motility, electromotility and lateral wall stiffness in the isolated outer hair cells. Hear Res 207(1-2), 68-75, 2005. [IF.: 1,67]
3. Sziklai I., Borkó R., Batta J.T. Potassium induced slow and fast motility changes in isolated outer hair cells of the guinea pig. Proceedings of the 5th International Symposium on Meniere's Disease & Inner Ear Homeostasis Disorders 162-164, 2005.
4. Borkó R., Batta J.T., Sziklai I. A lassú motilitás okozta oldalfal merevség fokozódás hatása az elektromotilitásra izolált külső szőrsejtekben. Fül-Orr-Gégegyógyászat 52(2), 94-103, 2006.

Indexed abstract

1. Borkó R., Batta J.T., Sziklai I. Correlation between slow motility, fast motility and lateral wall stiffness in the outer hair cells of the guinea-pig. Otolaryngol. Hung. 50, 199a, 2004

Lectures and posters

1. Sziklai I., Borkó R., Electromotile performance of isolated outer hair cells in different stages of slow motile shortening. Collegium Oto-Rhino-Laryngologicum Amicitiae Sacrum, Salvador, Bahia, Brazil. 22-25. 08. 2004.
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3. Borkó R., Batta J.T., Sziklai I. Correlation between slow motility, fast motility and lateral wall stiffness in the outer hair cells of the guinea-pig. Inner Ear Biology Workshop. Debrecen, 05-07. 09. 2004.

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