PhD THESES

THE STIFFNESS REGULATION OF THE MAMMALIAN OUTER HAIR CELL LATERAL WALL AND THE COCHLEAR AMPLIFIER

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2004

INTRODUCTION

Slow and fast shape changes of the outer hair cells (OHC) are key variables in the processing of the auditory signal at the periphery. As these shape changes represents the effector arm of the cochlear amplifier these are intensively examines experimentally. The membrane voltage dependent electromotility (fast motility) is generally considered as the basis of the local force feedback process within the cochlear partition, called cochlear amplifier. The slow motility of OHCs is appreciated as an adaptive and protective mechanism, which can keep the cochlear amplifier under control. Different constituents of the lateral wall can provide the lateral wall with different mechanical features which may play an important role in the electromechanical force coupling during cell shape changes (slow and fast motility). Mammalian outer hair cells have a unique trilaminated lateral wall composed of plasma membrane, cortical cytoskeleton and subsurface cisternae. The actin based cortical cytoskeleton is closely associated to the subsurface cisternae and connected to the lateral plasma membrane whereas it is replaced by the subsynaptic cisternae at the basal (infranuclear) region. At the apical (infracuticular) segment of the OHCs there is a collection of dense filaments. The motor mechanisms work against the mechanical load of the lateral wall thus the stiffness of the OHCs' lateral wall presumably has an important role in the regulation of the cochlear amplifier. According to the membrane bending model described by Raphael et al. (2000) the constituents of the lateral wall can form micromechanical units, which can act as a motor protein independent lateral wall stiffness regulation system. Such regulation system can control the efficacy of the force feedback of the OHCs to the basilar membrane, which depends on the mechanical load against the motor proteins' work. Part of this load is generated by the overall cell stiffness of the OHCs therefore stiffness changes may provide an intrinsic mechanism in dynamic adaptation of the organ of Corti to actual requirements.

Acetylcholine (ACh) and γ-amino-butyric-acid (GABA), the main neurotransmitters of

the efferent innervation of the outer hair cells, are assumed to regulate the efficacy of the cochlear amplifier through a variety mechanisms (e.g. regulation of the axial stiffness and the electromotile magnitude response of OHCs). The effect of ACh and GABA on the lateral wall stiffness and on the slow motility of OHCs, however, has not yet been documented.

ACh and GABA receptors show a tonotopic distribution along the cochlea in mammalians. Density of ACh receptors is higher in the basal turn OHCs and decreases significantly towards the apical turns. GABA receptors are cochleoapically biased in the cochlea. The inverse innervation pattern may be reflected in different sensitivities of OHCs to efferent neurotransmitters depending on their cochlear locations.

The stiffness of the OHCs has already been measured by microdeformation techniques. The axial and the transverse stiffness of OHCs are in linear correlation with the compression of the cell. The axial stiffness is smaller than the transverse one. This can well be explained by the cell's cylindrical shape and the structure of the cytoskeletal system. The axial stiffness of the cochleoapical and cochleobasal OHCs is reported to be different.

Very few measurements were designed to characterize the stiffness of the lateral wall of the OHCs. A high resolution stiffness mapping of the lateral wall and the comparison of the local stiffness in corresponding lateral wall regions in OHCs isolated from different cochlear locations have not been documented yet. A linear correlation has been already described between pulling force and maximal expansion of the lateral wall. This suggests that the expanded lateral wall segment behaves as an elastic material. The relationship between the pulling force and the deformation of the lateral wall is known, however, we do not know the characteristics of the lateral wall response to pulling force which can be described by a time-deformation function. This relationship can reveal the deformation response of the lateral wall to stretch whether it is merely a passive response or contains active components as well. The existence of a motor protein independent active response phenomenon of the lateral wall of

OHCs may give evidence for an intrinsic, dynamic, efferent neurotrasmitter controlled adaptation of the organ of Corti which issue has been risen by Fridberger et al. (1998).

The aim of this study are:

i., to produce a pseudocolour image analysis method, which increases the accuracy of the determination of the lateral wall stiffness.

ii., high resolution stiffness mapping of the lateral wall and the comparison of the local stiffness in corresponding lateral wall regions in OHCs isolated from different cochlear locations.

iii., to examine ACh and GABA-induced stiffness responses of OHCs isolated from cochleobasal and cochleoapical locations and the influence of these neurotransmitters on stretch-induced slow cell shortening.

MATERIALS AND METHODS

Preparation of cells

Pigmented, young guinea pigs of both sexes with positive Preyer's reflex were euthanized with pentobarbital. The organ of Corti was removed and incubated at room temperature with collagenase (type IV Sigma, 1 mg/ml) for 10 minutes. The cells were dissociated by a gentle mechanical trituration. OHCs were separately isolated from the cochlear apex and base. Cell length of OHCs were 26 ± 5 μm (n=47) and 74 ± 3 μm (n=54) for cochleobasal and the cochleoapical OHCs, respectively. The regular extracellular solution contained (in mM): 136.75 NaCl, 5.36 KCl, 0.34 Na_2HPO_4, 0.44 KH_2PO_4 , 0.81 $MgSO_4$, 1.26 $CaCl_2$, 5.56 glucose, 4.17 $NaHCO_3$, 10 HEPES (pH 7.4, 300 mOsm/l). Calcium-free extracellular solution was prepared by substituting Ca^{2+} with equimolar Mg^{2+} and adding 5mM EGTA.

Measurement of the lateral wall stiffness, cell visualization

Stiffness of the lateral wall was measured by the glass micropipette aspiration technique (Oghalai et al., 1998). Glass micropipettes were pulled from Clark EC 15 TF capillaries in 5 stages (Flaming/Brown Model P-87). The inner diameter of the pipette tips was 2.8±0.2 μm. Pipettes were filled with extracellular solution and connected to the pressure application apparatus through a polyethylene plastic tube. We have determined experimentally the pressure threshold required to detach the membrane form the cortical cytoskeleton and subsurface cisternae in our system. This threshold pressure was, in accordance with the results of Oghalai et al., (1998), between 12 and 15 cm H_2O. Therefore aspiration pressure ranging from 4 to 10 cm H_2O (0.39 nN/μm^2 -0.98 nN/μm^2) was applied to study the full trilaminated lateral wall using a calibrated water column. OHCs were placed in extracellular solution containing recording chamber (200 μl) and viewed under a Nikon inverted microscope using 400x magnification. Phase-contrast images of the aspirated lateral wall segments in the pipette were video-recorded, digitized and analyzed off-line after pseudocoloration. A CCD camera (Sony DXC-107P) and a PC connected external digitizing card (Pinnacle Systems, Inc. USA, California) were used to grab images (24 bit). The resolution of the digitized images was 18 pixels/μm. The projection of the tip of the pipette was chosen as the reference line. A line parallel to the reference line and tangent to the lateral wall curvature was drawn. The distance between these lines was determined and considered as the length of the aspirated lateral wall segment. A custom-made colour deepth analyser software was used to identify reference points after edge-detection, and noise filtering. The stiffness parameter (S_p) was calculated from the length of the aspirated lateral wall segment as follows:

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

where ΔP (nN/μm^2) is the negative pressure applied through the micropipette; r (μm) is inner radius of the micropipette; ΔL (μm) is length of the aspirated lateral wall (Oghalai et al. 1998).

The lateral wall of the OHCs' was classified into the following regions: infracuticular or apical region, midlateral region (from infracuticular region down to the upper edge of the nucleus). The midlateral region includes a small region right above the nucleus, the near-supranuclear region. We defined the infranuclear region from the lower edge of the nucleus toward the synaptic pole. Apical, midlateral, near-supranuclear and infranuclear lateral wall regions were examined in the same cell with the same pipette.

Application of test solutions

Sodium salicylate and GdCl$_3$ (Sigma) solutions were prepared at a concentration of 5 mM (pH 7.4, 300 mOsm/l). The isolated OHCs were exposed to one of these test solutions for 10 minutes in the recording chamber. Time course of the lateral wall stiffness under constant negative pressure (6 cm H$_2$O) was measured for 2 minutes before the exposition of salicylate or gadolinium and during the last 2 minutes of the exposure time. Influence of ACh and GABA were examined in OHCs isolated separately from the apex and base of the cochlea. Strichnine as ACh antagonist and bicuculline as GABA$_A$ receptor inhibitor were used, all in 50 μM concentrations. Bicuculline was prepared in a 1 mM stock solution in dimethyl sulfoxide (DMSO) and was diluted by the external solution to the final concentration of 50 μM. The final concentration of DMSO in the extracellular solution was less than 0.1% which has no influence on the active and passive behavior of the stiffness of OHC's lateral wall. Isolated OHCs were exposed to only one of the test solutions for 4 minutes in the recording chamber using a manually controlled, gravity driven microflow system with low flow rate

(0.6 µl/min.). Time course of the lateral wall stiffness under constant negative pressure (6 cm H$_2$O) was measured during the last 2 minutes of the exposure time. In the control experiments we used microflow of the normal incubation solution. One cell was measured under the influence of only one of the test solutions.

Data analysis
We compared S$_p$ and the parameters characterizing the regulatory stiffness response determined for 27 cells isolated from 5 animals. Statistical analysis (ANOVA) showed that cells isolated from different animals can be considered as a homogenous population regarding the measured parameters ($p>0.5$). Based on this result we made the assumption that cells isolated from different animals will respond similarly to a given treatment. Therefore we pooled the data obtained in response to the same treatment recorded in OHCs isolated from different animals. The subsequent statistical analysis was ANOVA, and multiple comparisons versus control group (Dunnett's method) were applied when significant difference was observed between the treatment groups (n=10 in each group). For the comparison of two independent groups, unpaired t-test was used. Differences were considered significant at $p<0.05$. Changes in the fitted parameters in eq.2 were reported in percent (%) in order to emphasize the responses of cochleoapical and cochleobasal OHCs to different treatments. Eq. 2 was fitted separately to the data points obtained on 10 individual cells per treatment. Percent change was calculated as [(mean-mean$_{control}$)/mean$_{control}$]×100, where mean and mean$_{control}$ are the average of the parameters in the treatment group and in control group, respectively. The reported errors for % changes were calculated using the Gaussian error propagation method. A similar approach was used to characterize stretch-induced slow cell shortening.

RESULTS

Characterization of the stiffness parameter in different regions of the OHC lateral wall.
　　　In order to characterize the relationship between the applied negative pressure (ΔP) and the length of the aspirated lateral wall (ΔL) we applied 30-second-long negative pressure

pulses ranging from 4 to 10 cm H_2O separated by 30 seconds relaxation periods. ΔL was determined at the end of the negative pressure pulse and plotted as a function of ΔP. A good linear correlation was found (r=0.91; p<0.001) between negative pressures applied and the length of the aspirated lateral wall (ΔL-ΔP), similarly to other reports. S_p is different at the ciliary pole, at the lateral wall and at the synaptic pole ranging from 1.83±0.13 nN/μm to 1.14±0.16 nN/μm, (mean±SD). The stiffness of the lateral wall is greatest at the infracuticular plate region, lower throughout the midlateral region and lowest in the infranuclear region.

Stiffness parameter in OHCs isolated from different regions of the cochlea

The stiffness of the lateral wall of the OHCs was mapped along the cell length by measuring the ΔL at constant 6 cm H_2O negative pressure. The length of the aspirated lateral wall segment was relatively constant for the first ~12 μm, followed by a shallow increase to a constant value throughout the midlateral region toward the nucleus. The stiffness of the lateral wall around the nucleus could not be determined using our experimental apparatus since the length of the aspirated lateral wall segments were too small to be measured accurately. This means that stiffness of the lateral wall is very high in this region. The length of the aspirated lateral wall segment at 6 cm H_2O negative pressure was constant in the synaptic region, the calculated S_p value indicates that the stiffness of this region is the smallest among the regions studied.

The length of the aspirated lateral wall shows a qualitatively similar dependence on the distance from the ciliary pole for cochleoapical and cochleobasal cells, including the nuclear and synaptic regions. Due to the difference in the length of the OHCs isolated from different cochlear locations the positions along the lateral wall were expressed as a percent of the total cell length from the ciliary pole. Application of this transformation yields virtually superimposable ΔL–position (%) relationships. There is no difference between the stiffness parameter in corresponding lateral wall positions (%) of cochleobasal and cochleoapical

OHCs. The stiffness of the OHC lateral wall features an abrupt change at a distance 15-20% from the cuticular plate, regardless of the cochlear location of the examined OHCs.

Time dependent change in lateral wall stiffness suggests active and passive behavior

The change in length of the aspirated lateral wall as a function of time (ΔL-ΔT) shows similar relationship in all 3 supranuclear lateral wall locations measured. The differences between the curves are quantitative and are due to the greater stiffness of the lateral wall close to the cuticular plate. Conversely, ΔL-ΔT curves in the infranuclear region differ qualitatively from those of supranuclear curves. The ΔL-ΔT curves in the infranuclear region show a simple exponential relationship, whereas those derived from supranuclear regions include a sigmoid component between 30 to 60 seconds after the application of negative pressure. Negative pressure also evoked a slow shortening of the cells. This shortening obtains its peak velocity of ~0.03 $\mu msec^{-1}$, between 30-60 second of application of negative pressure. Neither the magnitude of the sigmoid component nor cell shortening showed significant correlation with the length of the cell. The slow cell shortening phenomenon was not observed when OHCs were incubated in a calcium-free medium (n=10). Incubation in calcium-free medium increased the stiffness of the lateral wall significantly from 1.35 nN/μm to 1.99 nN/μm (p<0.003). In control experiments we determined that 2 mM Mg^{2+}, which was used to substitute Ca^{2+} in calcium-free medium, does not affect the stiffness of the later wall. The stiffness parameters were 1.35±0.23 nN/μm and 1.27±0.2 nN/μm in normal bath solution (0.81 mM Mg^{2+}) and in 2 mM Mg^{2+}-containing solution, respectively (n=5). The preservation of the active stiffness regulation of the lateral wall (sigmoid component, see discussion) in a calcium-free medium may be indicated by a barely visible break point on the ΔL-ΔT curve at 50 second which is the time point of the active lateral wall response of OHCs when incubated in calcium-containing fluid. The ΔL-ΔT curve of the infranuclear region maintains its behavior in the absence of calcium.

Modeling passive behavior of the OHC lateral wall

The length of the aspirated lateral wall segment as function of time *L(t)* can be modeled as follows. We define the elementary elastic units as the complex of the plasma membrane and the associated cytoskeleton between adjacent pillar proteins. In our model these units can be in a resting or expanded state at a given negative pressure, and the transition from the resting to the expanded state follows a first order kinetics with negligible reverse reaction rate. Assuming that in the absence of negative pressure at t=0 all units are in the resting state, the whole length of the aspirated lateral wall segment as function of time (*L(t)*) is given by

$$L(t) = a \cdot (1 - e^{-b \cdot t}) \qquad (2)$$

where *a* is the maximum possible length of the aspirated lateral wall (i.e. the product of number of all elastic units (N_0) and the length of the elastic units (*l*) in the equilibrium expansion state), *e* is the natural number and *b* is the lateral wall expansion velocity constant. Taking into consideration the slow sliding of the lateral wall, subsurface cisternae and the cytosol into the pipette the length of the lateral wall segment without active behavior as a function of time is:

$$L^*(t) = a \cdot (1 - e^{-b \cdot t}) + m \cdot t + k \qquad (3)$$

where: *m* is the lateral wall sliding modulus, *k* is the viscosity coefficient, *t* is time, all other variables were defined above. The average sliding modulus determined from fits to the experimental data points using this model was 0.00022 μm/s, resulting in a 0.026 μm sliding into the pipette over the period of 120s used in our experiments. Thus, sliding of the membrane into the pipette is negligible, it does not influence our conclusions.

Modeling active behavior of the lateral wall

In the subsequent sections we attributed the sigmoid component of the ΔL-ΔT curve to an active stiffness regulation of the lateral wall. Since the elementary elastic units of the lateral wall (see above) can work not only as voltage sensitive elementary motor units, we modeled the sigmoid component as an active shortening of the aspirated lateral wall. Our model is based on the assumption that microdeformation-induced strain in the membrane will activate stretch-gated channels and the consequent Ca^{2+} influx will drive the shortening of the elastic units.

The time-dependence of the lateral wall's strain ($\sigma(t)$), which may regulate the activity of stretch-gated channels, can be described as:

$$\sigma(t) = \frac{F_t}{A} = \frac{F_p - M \cdot \frac{(a \cdot (1 - e^{-b \cdot t}) + m \cdot t + k)}{t^2}}{A} \qquad (4)$$

where: t is time; F_p is pulling force; F_t is tension force; M is the inertia moment of the aspirated lateral wall segment; A is the area of the aspirated lateral wall segment; other variables are defined above.

With respect to the absolute and relative value of F_p (about 3×10^{-9} N) and the length of the aspirated lateral wall segment (about 2×10^{-6} m), the time dependence of the strain is a shallow exponential curve with small slope and good linear correlation in the 0-120 second range. Considering this and the instantaneous response of the stretch gated ion channels to their activating signal the time dependence of the number of active contraction units should be described by a Boltzmann function. Consequently, the sigmoid component of the ΔL-ΔT curves in the supranuclear lateral wall regions can be modeled as:

$$L_c^*(t) = a \cdot (1 - e^{-b \cdot t}) - \frac{c}{1 + e^{-\frac{t-t_0}{d}}} + m \cdot t + k \qquad (5)$$

where 'c': maximum active retraction of the aspirated lateral wall segment; 'd': lateral wall area retraction velocity constant; t_0: half time of the whole process. Fits to the experimental data sets using Eq. 5.

The effect of salicylate and gadolinium ions upon the active and passive behavior of the lateral wall

Salicylate is known to inhibit prestin-mediated motility of the OHCs. To reveal whether the complex response of the lateral wall observed in this study is influenced by this motor protein we compared the ΔL-ΔT curves in the absence and presence of salicylate. There was no significant change either in the active or the passive characteristics of the ΔL-ΔT curves when salicylate (5 mM) was applied to the OHCs (n=10). In order to study the involvement of cation channels in the stiffness regulation of the lateral wall, a non-specific cation channel blocker, gadolinium (5 mM) was applied to the incubation fluid. Application of gadolinium induced a complex change in the ΔL-ΔT curve as compared to control. The most important change was the marked decrease or even disappearance of the active stiffness regulation (diminished sigmoid component). The passive behavior of the lateral wall was influenced by gadolinium treatment, which is manifested in the downward shift of the ΔL-ΔT relationship (n=10). Using our mathematical model (Eq. 5) we determined separately the effect of Gd^{3+} on the passive and active component of the ΔL-ΔT relationship. Our results indicate that both of these components are significantly altered upon Gd^{3+} treatment (p<0.05). Negative pressure evoked slow shortening of the cells was also inhibited by Gd^{3+} treatment. The maximal cell shortening was 0.85±0.23 μm in control solution and 0.5±0.18 μm during Gd^{3+} treatment (n=10). In summary, gadolinium, as a nonspecific cation channel blocker, attenuated the active stiffness regulation characteristics, increased the stiffness of the lateral wall and inhibited the negative pressure evoked slow shortening of the cells.

The effect of ACh and GABA on the stiffness of the OHC lateral wall of different cochlear location

Under control conditions, there was no significant difference between the S_p of cochleobasal and cochleoapical cells (t-test, p=0.70, df=18). Influence of ACh (50 µM) and GABA (50 µM) on the stiffness of the OHC lateral wall was dependent on the localization of the OHCs along the cochlea. The stiffness of the lateral wall was significantly decreased when ACh was given to the incubation medium of cochleobasal OHCs, whereas GABA was ineffective (ANOVA followed by multiple comparisons versus control group (Dunnett's Method), p<0.05 for significant differnece). A similar statistical comparison for cochleoapical OHCs showed that the stiffness of the lateral wall was significantly decreased by GABA; the effect of ACh was statistically not significant. The effects of ACh and GABA on the lateral wall stiffness of cochleobasal and cochleoapical cells, respectively, were not significantly different (multiple comparisons versus control group (Dunnett's Method), p>0.05). There was no significant change in the stiffness when 50 µM strychnine, the specific inhibitor of the α9 ACh receptors expressed in OHCs, was used in combination with ACh in the incubation medium of cochleobasal OHCs or when 50 µM bicuculline was used in combination with GABA in cochleoapical OHCs (multiple comparisons versus control group (Dunnett's Method), p>0.05).

Influence of ACh and GABA on the active and passive changes in lateral wall stiffness as a function of time

The stiffness behavior of the lateral wall during micropipette aspiration of OHCs can be derived from the length of the aspirated lateral wall segment in the function of aspiration time (ΔL-ΔT). The ΔL-ΔT function of the aspirated lateral wall has special characteristics which are independent from the cochlear location of the OHCs. The regulatory stiffness response between 30-60 second of the application of negative pressure increases the stiffness of the lateral wall. The effect of ACh and GABA on the ΔL-ΔT function is strongly related to

the cochlear locations of the OHCs. The effects of ACh and GABA on the stiffness regulation

of OHCs were interpreted based on the parameters of the ΔL-ΔT model function (Eq. 2) fitted

separately to the data points obtained on 10 individual cells per treatment. ACh increased 'a',

the maximum possible length of the aspirated lateral wall segment in the pipette by 50±12%

(n=10) and 'c', the maximum possible active retraction by 85.5±17% (n=10) and 'd', the

lateral wall area retraction velocity constant by 59.3±20% (n=10). Simultaneously, ACh

decreased the lateral wall stiffness significantly by 18.9%. These data were obtained in OHCs

isolated from the basal cochlear turn (average OHC length was 31±5 μm). GABA, similarly to

ACh, increased the maximum possible length of the aspirated lateral wall segment in the

pipette by 33±13% (n=10), the maximum possible active retraction by 47.6±12% (n=10) and

the lateral wall area retraction velocity constant by 24.5±16% (n=10) whereas it decreased the

lateral wall stiffness by 15.4% in apical turn OHCs (average OHC length was 76±4 μm). The

regulatory stiffness responses during micropipette aspiration are quantitatively different in the

presence of ACh and GABA. The effect of the ACh and GABA on the lateral wall stiffness

regulation can be inhibited by their potent antagonists strychnine and bicuculline.

ACh and GABA modify the characteristics of the slow cell shortening evoked by lateral wall microdeformation

Micropipette aspiration of the OHC lateral wall induces a slow cell shortening simultaneously

with an active stiffness increase of the lateral wall. In the presence of ACh (50 μM), the

magnitude of microdeformation induced shortening was increased by 87.7±19% (n=10) as

compared to control, whereas GABA (50 μM) increased cell shortening by 64.6±18% (n=10)

in cochleoapical cells (ACh and GABA do not induce cell shortening by themselves). The

shortening velocity constant was not changed by GABA treatment, unlike ACh, which

increases the shortening velocity constant by 42.8±12% (n=10), (Fig. 3.).

Effects of Ach and GABA in the absence of extracellular Ca^{2+}

In the presence of extracellular Ca^{2+} regulatory stiffness response is enhanced both by

ACh and GABA. In order to get insight into this phenomenon we measured the effect of ACh and GABA in the absence of extracellular Ca^{2+}. In the presence of ACh and GABA the regulatory stiffness response is preserved in no Ca^{2+} condition, although the magnitude of the active process is remarkably decreased as compared to the data obtained in the presence of Ca^{2+}. The maximum possible active retraction (parameter 'c' in eq. 2) was decreased in the absence of Ca^{2+} to 58.2±17% (n=10) and 53.1±17% (n=10) of the value determined in the presence of Ca^{2+} for ACh (50 μM) and GABA (50 μM), respectively.

DISCUSSION

The complex structure of the lateral wall of the OHCs endows it with special attributes. The structural elements of the lateral wall theoreically can allow the lateral wall to function as a mechanical regulator during active movement of the OHCs within the cochlea. Since the molecular motor units work against the mechanical load of the cell the active stiffness regulation of the lateral wall presented in this study can control the electromotility of the OHCs. In this function perhaps the most important element is the cortical cytoskeleton, which is an actin-based structure with long parallel filaments oriented approximately circumferentially and cross linked by fine fibrils associated to the cytoplasmic surface of the plasma membrane and the subsurface cisternae. The circumferential filaments of the subcortical cytoskeleton form discrete domains that can accommodate large shape changes. The special structure of these microdomains probably makes possible the transformation of the cumulative action of the elementary, independently activated, voltage-sensitive motors along the lateral wall to shape changes of the cells. The lateral wall of the OHCs is under tension by the cortical cytoskeleton, which presents an elastic preload of the cell.

The elongation of the lateral wall of the OHCs' as a function of force during micropipette aspiration is similar to an elastic deformation regardless of the position along the

longitudinal axis of the OHCs. However, the stiffness of the lateral wall along the longitudinal axis of the cell is not constant. Our results show a similar apico–basal stiffness difference in OHCs isolated from different cochlear locations. Iso-stiffness lateral wall positions are found at the same position as a proportion of the cell length. This means that the corresponding lateral wall segments having the same Young's modulus constitute the same fraction of the total cell length regardless of the cochleoapical or cochleobasal origin of the cells. Since the length of the cells increases toward the cochleapical direction the absolute length of the iso-stiffness lateral wall segments increases too. Thus, according to our results, longer (apical) OHCs are composed of proportionally longer cylinders than shorter (basal) ones, but the stiffness (and Young's modulus) of the corresponding cylinders is the same. Our results apparently contradict earlier observations where smaller axial stiffness was reported for longer OHCs than for shorter ones. However, if we take into consideration Euler's beam theory, where the axial striffness of a segment is reversely prorprional to its lenght, at identical Young's modulus longer cylinders will have smaller axial stiffness. Thus, the cumulative axial stiffness of the cylinders, i.e. the axial stiffness of the OHCs, decrease with increasing cell length, in accordance with the literature.

The lateral wall of the OHCs also shows an active behavior during microdeformation in a close association with a synchronized slow cell shortening.

The active cell-shortening and active lateral wall-behavior during micropipette aspiration does not seem to be explainable merely by the mechanoelectric phenomenon associated with the lateral wall strain. According to the mechanoelectric theory stretching the membrane results in a hyperpolarization whereas mechanical compression results in a depolarization of the membrane. Change in the OHCs' membrane potential is represented unambiguously in the length of the cells. Straining of the aspirated lateral wall segment evokes hyperpolarization and mechanical relaxation as a consequence of the elongation of the

motile units. This is in clear contrast to our findings, where we reported slow cell shortening upon micropipette aspiration of the lateral wall.

Prestin-mediated membrane potential-dependent changes in the OHC's length were described recently by Dallos and co-workers. This mechanism seems to be responsible for oscillation in the OHC's length driven by the frequency of the sound. Based on the following, we exclude this mechanism for the explanation of our results: *i*, the prestin mediated response is sensitive to salicylate whereas the slow cell shortening and stiffness changes reported in this study were sensitive to gadolinium ions but insensitive to salicylate. *ii,* The magnitude of the prestin mediated cell shortening is not similar to our results and the time domain is in the ms-µs range whereas slow cell shortening reported in this study takes place over 30-60 s. *iii,* Slow changes in OHC length are dependent on extracellular Ca^{2+} , whereas the prestin-mediated response does not exhibit a dependence.

One mechanism, which might be responsible for our results, is compatible with the membrane bending model where the elementary motors and elastic units are defined as the complex of the plasma membrane and the associated cytoskeleton between adjacent pillar proteins. Depolarization results in shortening whereas hyperpolarization results in elongation of the cells in this model. Assuming that the force applied to the lateral wall is much higher than the physiological range, thus, activation of stretch activated non-selective ion channels may induce a sufficient depolarization of the membrane for evoking whole cell shortening. Indeed, non-selective cation channels exist in the membrane of OHCs which can depolarize the membrane and induce slow cell shortening. Stretching the lateral wall under our experimental conditions can induce opening of similar channels. Supporting this scenario gadolinium, a substance known to block stretch-activated channels, inhibits slow cell shortening and decreases significantly the sigmoideal component of the ΔL-ΔT function. Takahashi and Santos-Sacchi. (2001) have mapped stress induced gating currents along the length of the

OHC. They described that the current is high in the midlateral region and decreases towards the cell extremes. Lanthanoids decreased the current but did not abolish it. This observation is similar to our finding. The active component of the ΔL-ΔT curve at the midlateral region is greater than at the near extremes and gadolinium treatment decreases the active part but does not abolish it.

An additional explanation of our results can be a calcium-dependent metabolic modification in the cell membrane or subcortical lattice (e.g. phosphorylation-dephosphorylation), which may modify the stiffness of the lateral wall. Minamino and co-workers (1998) studied slow cell shortening induced by tetanic electrical field stimulation. They found that cell shortening occurs 20-30 s after the stimulation and the duration of the response is 1-2 min., in agreement with our experimental findings. Blocking the Ca^{2+}-calmodulin-CAM kinase pathway by W-7 and KN-62 inhibited slow cell shortening, similar to our results obtained in calcium free medium and in the presence of 5 mM gadolinium. These results point toward the presence of a Ca^{2+}-calmodulin-CAM kinase dependent contraction machinery in OHCs. Our results also showed that both gadolinium treatment and the absence of extracellular Ca^{2+} increased the stiffness of the lateral wall. Thus, besides the lack of activation of a putative Ca^{2+}-dependent contraction mechanism the increase in the stiffness of the lateral wall might also contribute to the inhibition of cell shortening under these conditions. The Ca^{2+}-dependence of the stiffness of the lateral wall is unclear yet, but it may include a Ca^{2+}-calmodulin-protein phosphatase pathway.

Micromechanical units similar to those in supranuclear regions are missing in the synaptic region. However, passive membrane characteristics of supranuclear and synaptic regions are qualitatively similar. Our theory is that constituents of the plasma membrane (cholesterol, proteins, etc.) provide the infranuclear membrane with a significant stiffness and

may generate quasi elastic units with the subsynaptic cisternae responsible for the passive behavior of the basolateral wall.

We successfully modeled both the passive and active behavior of the lateral wall during micropipette aspiration using simple laws of physics. Our excellent fits to the experimental data showed that the time dependence of the length of the aspirated lateral wall segment follows an exponential relationship. This model was based on the distribution of the elastic units, formed by the constituents of the lateral wall, between two length states, thus, the lateral wall cannot be considered as a simple elastic material. This two state model was expanded by an active shortening component to account for the complex ΔL-ΔT function measured in the supranuclear regions. Although we do not know the molecular basis of the putative contraction units yet, the good fits to the experimental data predict the existence of such units, which reach equilibrium activation instantly following the activation of stretch receptors in the membrane.

In summary, the stiffness of the lateral wall of the OHCs is under active regulation, it is tightly linked to slow cell shortening and might also influence harmonic cell shape changes induced by the acoustic signal. The importance of Ca^{2+} and the operation of stretch gated ion channels in the regulation of lateral wall stiffness and cell shortening was shown in this study. This opens the possibility for the modulation of OHC functions by Ca^{2+}-dependent phosphorylation-dephosphorylation reactions; thereby cell length changes may become sensitive to neurotransmitters released by efferent neurons. Albeit the role of the active cell response phenomenon presented here is not clear in the dynamic regulation of the force feedback to the basilar membrane driven by the electromotile activity of the OHCs, our findings can present additional prospects to consider in adaptation, automatic gain control and non-linear properties of the cochlea This electromotility independent intrinsic regulation system of OHCs, which can change the stiffness of the lateral wall (regulatory stiffness

response) and the length of the cell in tight association with Ca^{2+} is a novel capability for controlling the mechanical preload of the OHCs and its electromotile activity dependent force feedback to the basilar membrane. ACh and GABA, the main neurotransmitters of the efferent innervation of OHCs, may regulate this . The characteristic effects of these neurotransmitters were a decrease in the stiffness of the lateral wall and an increase the regulatory stiffness response and the magnitude of the microdeformation induced slow cell shortening. In accordance with the literature, we found a cochleobasally biased ACh-response and a cochleoapically biased GABA-response in the lateral wall stiffness and its regulation: the effects of ACh and GABA were exclusively present in basal and apical turn OHCs, respectively; they were ineffective in the reciprocal locations.

Dallos et al. (1997) suggested that ACh-mediated fast motility response increase of the OHCs is a consequence of a decreased axial stiffness of the cells. Our data suggests that a similar relationship exists between the stiffness of the lateral wall, the regulatory stiffness response and stretch-induced slow cell motility: efferent neurotransmitter-mediated decrease of lateral wall stiffness increases the active responses of the cells (i.e. regulatory stiffness response of the lateral wall and the stretch induced slow cell motility).

The role of calcium as a second messenger mediating the ACh influence on stiffness and fast motility was described earlier. ACh was reported to open big conductance cation-selective channels: nicotinic α_9/α_{10} ACh-receptors, in the synaptic region of the OHCs. A close correlation between extracellular Ca^{2+} concentration and ACh effectiveness upon lateral wall stiffness and regulatory stiffness response is also demonstrated in the present paper.

A bicuculline-sensitive GABA-mediated Ca^{2+} -signaling was described in cerebellar Purkinje cells which develops by activation of voltage-dependent Ca^{2+} -channels associated to GABA receptors. The membrane potential shift is induced, in this case, by a HCO_3^- efflux through GABA receptor channels. A similar mechanism might also be responsible for the

influence of GABA on cochleoapical OHCs. A putative GABA-mediated Ca^{2+} influx may result in a same process in cochleoapical OHCs as may an ACh-mediated Ca^{2+} -influx in the basolateral cells. This would be in accordance with the similarities between the biased effect of ACh and GABA on the lateral wall stiffness in OHCs of different cochlear locations. There are, however, dissimilarities between the influences of the two neurotransmitters. Effect of ACh is greater on all examined parameters in cochleobasal OHCs than those of GABA in cochleoapical OHCs. GABA modifies different aspects of the stiffness-related behavior of the lateral wall (maximum possible length of the aspirated lateral wall, lateral wall area retraction velocity constant) and associated slow shortening than does ACh. Mechanism of GABA responsiveness in Purkinje cells may also help to understand these differences. ACh-receptors permit Ca^{2+} influx directly whereas Ca^{2+} -influx is triggered indirectly by GABA. The latter may activate a Ca^{2+} related cellular signaling of smaller magnitude.

Incubation of isolated OHCs in Ca^{2+} -free medium results in a stiffness increase (47%) of the lateral wall and a virtual disappearance of the active regulatory stiffness response. Basal turn OHCs respond to ACh with attenuated stiffness increase and preservation of the regulatory stiffness response in the absence of extracellular Ca^{2+}. According to our expectations, the magnitude of the regulatory stiffness regulations is reduced in the absence of extracellular Ca^{2+}. Similar results were obtained in Ca^{2+} free medium for apical turn OHCs in the presence of GABA.

One of the putative consequences of elevated intracellular Ca^{2+} is phosphorylation of cytoskeletal proteins and prestin. This is also suggested by an increase of viscosity coefficient in Ca^{2+} -free medium and after Gd^{3+} treatment as calculated from experimental data, as well as by the fact that no significant difference is observed in the effect of ACh and GABA on the passive stiffness behaviour. As it has been previously described, the microdeformation-

induced lateral wall stiffness changes and slow motility are independent of prestin and are related to the cytoskeleton.

Based on our results our idea is that that efferent innervation protects the organ of Corti against high sound intensities and support adaptation indirectly, via the potentiation of stretch induced cell shortening, rather than by a direct stiffness regulation-related mechanism. Loud sounds vibrate the basilar membrane with greater amplitudes, which might result in a greater stretch of the lateral wall of OHCs thereby stimulating slow –quasi static– shortenings of the cells leading to adaptation. Reduced stiffness of the lateral wall in the presence of the efferent neurotransmitters and the consequent increase of motile capability of the cells could potentiate this stretch-induced shortening in the presence of Ach (cochleobasal OHCs) or GABA (cochleoapical OHCs). Cell shortening increases the lateral wall stiffness, the axial and transverse stiffness of the cells. This stiffness increase can be sufficient in magnitude to change micromechanical properties of the cochlear partition to compete with loud-sound induced distorsion of the organ of Corti.

As a conclusion, efferent neurotransmitters increase responsiveness of OHCs against mechanical insults via a robust, prestin-independent cell shortening accompanied by increase in the cell stiffness. These changes may sufficiently modify the mechanical properties of the organ of Corti to damp vibrations and consequently protect the cochlear partition. This is in a good agreement with the observation of Friedberger et al. (1998) who described a ~6 μm dynamic contraction of the organ of Corti of the guinea pig simultaneously with intracellular Ca^{2+} concentration increase of OHCs induced by noise stimulation.

Publications that formed the base of the PhD theses:

1. Batta J.T., Panyi Gy., Gáspár R., Sziklai I. Active and passive behaviour in the regulation of stiffness of the lateral wall in outer hair cells of guinea pig. Pflüg. Arch. Eur. J. Physiol. 447:328-336, 2003. [IF.:2]

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3. Batta J.T., Panyi Gy., Gáspár R., Sziklai I. Tengerimalac cochleaból izolált külső szőrsejtek oldalfalmerevségének aktív szabályozása, mint a cochleáris erősítő része. Fül-,Orr-, Gégegyógyászat 50:133-141, 2004

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6. Borkó R., Batta J. T., Sziklai I., Lassú sejthosszváltozás, oldalfalmerevség és elektromotilitás tengerimalac izolált külső szőrsejteken. Magyar Fül-Orr-Gégeorvosok Egyesülete Audiológiai Szekció Vándorgyűlése, Debrecen, 1-3 09.2004.

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Other publications:

1. Borkó R., Batta J.T., Sziklai I. Electromotile performance of isolated outer hair cells during slow motile shortening. Act. Otolaryn.in press 2005. [IF.: 0.8]

2. Borkó R., Batta J.T., Sziklai I., Slow motility, fast motility and lateral wall stiffness in the outer hair cells of the guinea pig. Hear.Res. under publications 2005. [IF.: 1.5]