

THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY (Ph. D.)

The role of purinerg signaling and Deiters cells in the inner ear

by

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1. INTRODUCTION

1.1. The physiological mechanism of hearing

Mammals are capable of perceiving high frequency sounds due to an active mechanism in the OHCs. The role of outer hair cells (OHC) in the amplifier mechanism of the cochlea at low intensity sound signals is well described, and is assumed to be mediated by the prestin-driven fast motility of the OHCs. According to former experiments this amplifier mechanism is influenced by different neurotransmitters such as acetylcholine (Ach) and GABA. Ach can change the electromotile activity of OHCs via an increase in the intracellular calcium concentration, which may cause phosphorylation of membrane or cytoskeletal proteins. This mechanism could then lead to reduced axial stiffness and a subsequent increase of the electromotile response. The contribution of the Deiters cells to the active mechanism of the cochlea have not yet been described. The presence of nerve fibers and terminals among Deiters and Hensen cells of the organ of Corti of the adult guinea pig could be detected using immunohistochemical methods. Among the supporting cells, the Deiters cells are in close contact at their apical and at the basal pole with outer hair cells. *In situ*, the cell bodies of the OHCs are positioned between the cell bodies and apices of the phalangeal processes of the Deiters cells. The length of the OHCs, and therefore the length of the phalanx of the Deiters cells' processes depend on their position in the organ of Corti. Short (long) OHCs are adjacent to short (long) phalangeal processes in situ. The phalangeal process covered by the phalangeal head possessing a larger diameter than the phalanx itself. The length of these two important cell types depend on their position in the organ of Corti, from base to apex, and from the first to the more lateral third-row. This morphological heterogeneity may lead to functional diversity, and via this mechanism, may play an important role in the perception of different sound frequencies.

1.2. The role of ATP in the inner ear

Beside the two main efferent neurotransmitters, such as acetylcholin and GABA, ATP can influence the intracellular Ca^{2+} homeostasis. ATP can be found at concentrations of 13.0 ± 2.4 nM in the endolymph and 10.5 ± 3.9 nM in the perilymph with no significant difference between the two fluids in this respect.

Application of ATP to the guinea pig outer hair cell evokes a depolarizing cation current, which shows the pharmacological profile of a P_{2X} purinoceptor mediated current. Patch-clamp experiments revealed that extracellular application of 5-10 μ M ATP to OHCs induces an inward current that is reduced by both purinergic receptor antagonists, suramin (100 μ M) and cibacron blue (100 μ M). The effect of ATP on calcium currents of OHCs has been reported to be heterogeneous. Application of high concentration of ATP (100 μ M) decreased Ca^{2+} currents at 0 mV, enhanced the currents at +10 mV, and had little effect on the currents in a subpopulation of these cells. This heterogeneous nature of the ATP-induced Ca^{2+} currents may reflect a functional diversity, including different expression of purinoreceptor subtypes among OHCs. Indeed, with the use of deoxyadenosine 5'-(α -[^{35} S]thio)triphosphate and [^{3} H] α,β -methylene-ATP for autoradiographic labeling, both P_{2X} and P_{2Y} receptors were detected in the organ of Corti. On the other hand, the organ of Corti was labeled intensively with P_{2X2} antibodies. In another set of experiments strong immunolabeling of the P_{2X7} receptor protein was observed in the inner and outer hair cells of guinea pig in the early postnatal period, from birth to post natal day 6, and this declined with age. In former experiments reverse transcription polymerase chain reaction was performed and P_{2X} receptor subtypes were detected in the rat inner ear. In these experiments P_{2X2} , P_{2X3} , P_{2X4} , P_{2X7} , but not P_{2X1} , P_{2X5} , P_{2X6} mRNA were detected in the inner ear, and authors found slight differences in the expression of the P_{2X} receptor subtypes between the organ of Corti, the vestibular system, and the spiral ganglion. They also demonstrated the presence of two isoforms of the P_{2X2} receptors in isolated OHCs.

Previous functional studies revealed that ATP evoked changes in $[Ca^{2+}]_i$ are first detected at the apical end, and are then followed by a delayed rise at the basal part. This observation suggests a non homogenous distribution of P_2 receptors in the membrane of OHCs. Thus, in addition to the differences in the purinoceptor expression, polarized expression of these receptors within a single cell might also contribute to the heterogeneity of the biological response to ATP.

1.3. The role of purinergic receptors

Two main subclasses of purinergic receptors can be discriminated; the ionotropic P_{2X} receptors and the G-protein coupled metabotropic P_{2Y} receptors. These receptors can be further subdivided into P_{2X1-8} and $P_{2Y1,2,4,6,11,12}$ subpopulations. The existence of further isoforms has also been suggested. Within the P_{2X} receptor family, the P_{2X1} mediates a rapid

change in intracellular calcium concentration ($[Ca^{2+}]_i$) in response to ATP the amplitude of which does not decrease after repetitive addition of the drug. P_{2X2} receptors, on the other hand, are slowly activated and do not show desensitization. The kinetics of activation of P_{2X3} , P_{2X4} , P_{2X6} , P_{2X7} receptors are between the previous two. Since the P_{2Y} receptors are metabotropic receptors, cell activation through these receptors is slow.

1.4. The contribution of Deiters cells to the hearing sensitivity

The contribution of the Deiters cells to the active mechanism of the cochlea is still not clear. Previous experiments showed the involvement of Deiters cells in the micromechanics of the organ of Corti. Stiffness change of the phalangeal process of these cells can be in the background of this observation, which may help to the active micromechanics of the organ of Corti. Different neurotransmitters such as ATP can modify the stiffness of the Deiters cell's phalangeal process. Application of 100 μ M ATP to the cell body of isolated Deiters cells induced a small, reversible movement of the cells' stalk whereas application of control (ATP-free) bathing media did not. This property of Deiters cells may be an additional variable in the cochlear micromechanics. Other experiments revealed an active movement of the head of the phalangeal process of Deiters cells in response to calcium influx. They suggested that such calcium influx may be mediated by ATP and other neurotransmitter co-release.

Only few experiments were performed to clarify the electrophysiological background of the observations mentioned above. Using patch-clamp an outwardly rectifying, voltage-dependent, potassium selective current was detected. Using different potassium channel blockers (4-AP, TEA), this current seemed to be produced by two different types of potassium channels. These outwardly rectifying potassium channels in Deiters cells may restore the cells' resting membrane potential subsequent to depolarizing ion fluxes, e.g. P_{2X} receptor-related conductances. Depolarizing shifts in the resting potential may also be caused by the increase in the extracellular potassium in the tiny space between OHCs and Deiters cells.

1.5. Effect of noise on the hearing sensitivity

The influence of noise the hearing sensitivity is in the centre of the experiments in the field of inner ear research since many people suffer from its consequences. Sustained high-level noise often leads to the death of different cell types in the inner ear, consequently, leading to sensorineural hearing loss. Using morphological and functional examinations

(distortion product otoacoustic emission, DPOAE, and auditory brainstem response, ABR) neither hair cell damage nor changes in the OHCs' function were found after exposure to 81 dB SPL sound for 24 days. In contrast, exposure to high level noise (105 dB SPL for 72 h), both OHC loss and decreased amplitude of DPOAE were observed by the same authors. When using the lower intensity sound for sound conditioning before the use of the traumatic noise they found that the outer hair cell loss and the decrease in the amplitude of the OAE were significantly lower.

Among the cells of the inner ear, the outer hair cells (OHCs) are the principal targets of these noise-induced effects, as they are more sensitive to noise-induced damage than inner hair cells or pillar cells. High level noise exposure causes morphological changes, such as damage to stereocilia, changes in the intracellular structures especially the mitochondria, and swelling or loss of the OHCs. These degenerative changes occur mainly in the basal 20% of the organ of Corti. Subsequently, these changes can lead to cell damage, and alter the sensitivity of hearing.

Previous studies showed the modified viability and motility of outer hair cells isolated from high level noise-exposed (250 Hz, 125 dB SPL) guinea pigs. Subsequent experiments linked these changes to the Ca^{2+} entering through the surface membrane channels and the increased intracellular calcium level. Indeed, the increased intracellular Ca^{2+} level could initiate processes that may cause stiffness changes of the lateral wall of the OHCs and these stiffness changes may constitute an intrinsic mechanism in dynamic adaptation of the organ of Corti. This process may be responsible for the above mentioned motility changes. Noise exposure can also modify changes in the response to different neurotransmitters or neuromodulators, for instance to ATP. In these experiments, guinea pigs were exposed to moderate-intensity narrow band noise (cutoffs at 1.1 and 2 kHz; A-scale, rms: 65 dB SPL) 24 h a day for 10-11 days. There were significant differences in the incidence and magnitudes of cation currents evoked by ATP (100 μM) with a decreased response to ATP in short and an increased response in longer OHCs.

2. AIMS

2.1. Characterization of the relationship between ATP induced Ca^{2+} transients and the distribution of the purinergic receptors in the plasma membrane of outer hair cells of the inner ear

Using polyclonal antibodies, we aimed at localizing the subtypes of the two main classes of P_2 receptors in the OHCs of the guinea pig. The experiments were complemented with functional studies to describe the characteristics of ATP-evoked changes in $[\text{Ca}^{2+}]_i$. Along with identifying a number of P_{2X} and P_{2Y} receptors we also described a heterogeneous distribution of these receptors along the membrane of OHCs and two kinetically distinct responses in $[\text{Ca}^{2+}]_i$ upon the addition of extracellular ATP.

2.2. Effect of moderate noise therapy on the intracellular Ca^{2+} level and purinergic receptor expression in the outer hair cells of the guinea pig cochlea

Our aim was to examine the effect of noise (80 dB SPL, 14 days) on the intracellular calcium levels, on the expression pattern of purinoceptors in the membrane of the OHCs of the guinea pig, and characterize the concomitant changes in the purinergic signaling pathways upon ATP exposure. In addition to changes in the stiffness of the lateral membrane of these cells, these measured parameters were used to establish the connection between functional changes of the organ of Corti and noise exposure.

2.3. Characterization of voltage sensitive potassium currents in the membrane of the Deiters cells with different morphology

Considering the large variability in the morphology of Deiters cells and the expression of different K^+ channels, our aim was to analyze the biophysical and pharmacological aspects of K^+ currents of Deiters cells having different morphology.

3. MATERIALS AND METHODS

3.1. Cell preparation

Young guinea pigs of either sex (150-300 g) were anesthetized by Nembutal and decapitated according to the approved protocol of the Animal Care Committee of University of Debrecen (86/609/EEC). The temporal bones containing the bulla were removed and the bulla was opened to expose the cochlea. Each cochlea was placed into Hank's solution containing (in mM): 142 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂ (Sigma, St. Louis, MO, USA). The pH was adjusted to 7.4, the osmolality to 310 mOsm. The dissection of the organ of Corti and the isolation and digestion (Collagenase type IV, 5 minutes, 1 mg/ml; Sigma) of the cells were carried out in this extracellular solution. The outer hair cells were selected upon their normal morphological criteria.. These included (1) a cylindrical cell shape; (2) no swelling or other distortion of the cell membrane; (3) nucleus positioned near the base of the cell; (4) no Brownian motion of the organelles. Electrophysiological measurements were performed on those Deiters cells, which looked healthy, did not swell, the core of the cell situated in the middle portion of the cell body, and the cells had a curve shaped process. For the noise therapy examinations, we made two different preparations, one from the apical two turns and one from the basal turn of the cochlea.

3.2. Ca²⁺ fluorescence imaging

The measurements were carried out in the extracellular solution described above. Changes in [Ca²⁺]_i were monitored using Fura-2. To this end isolated cells were placed on a cover slip and loaded with 5 μM Fura-2 AM for 20 minutes. The cover slips with the Fura-2 loaded cells were then placed on the stage of an inverted fluorescence microscope (Diaphot, Nikon, Japan). Excitation wavelength was altered between 340 and 380 nm by a microcomputer-controlled dual-wavelength monochromator (Deltascan, Photon Technology International, New Brunswick, N.J., USA) whereas the emission was monitored at 510 nm using a photomultiplier at 10 Hz acquisition rate of the ratios. Intracellular [Ca²⁺] levels were calculated from the ratio of measured fluorescence intensities. Purinergic agonists were applied through a local perfusion system. The measuring bath was constantly perfused with normal HEPES Tyrode's solution (in mM, 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CaCl₂, 11.8 Hepes-NaOH, 1 g/l glucose, pH 7.4) at a 2 ml/min rate (Econo Pump, Bio-Rad Hungary, Budapest, Hungary). Test solutions were directly applied to the cells through a perfusion capillary tube (Perfusion PencilTM, AutoMate Scientific, San Francisco, CA, USA) with an

internal diameter of 250 μm at a 1.5 $\mu\text{l/s}$ rate, using a local perfusion system (Valve BankTM8 version 2.0, AutoMate Scientific, San Francisco, CA, USA).

3.3. Immunocytochemistry

After isolation, the cells were fixed with paraformaldehyde for 15-20 minutes. The cells were then incubated in a blocking solution of phosphate based saline (PBS) supplemented with 1% bovine serum albumin and 0.5% Triton X 100, for 30 min (Sigma). Subsequently the cells were incubated with polyclonal antibody raised in rabbit against the carboxy terminus of the rat purinergic receptors (Alomone Labs Inc., Jerusalem, Israel) for 90 minutes. The cells were then washed three times with PBS. The secondary anti rabbit antibody (raised in goat and labeled with fluorescein isothiocyanate; Vector Labs., Burlingame, CA, USA) was diluted 1:1000 in PBS. The cells were washed again three times in PBS, and then incubated with propidium iodide (1:1000; Sigma) to visualize the nuclei of the cells. After washing three times the specimens were covered with Vectashield (Vector Labs.). The slides were then observed under a fluorescent microscope.

3.4. Noise exposure

The intensity of the noise was set below the level thought to induce cellular damage based on former functional and morphological experiments. Guinea pigs were therefore exposed to continuous, moderate-intensity broadband noise (50 Hz - 35 kHz, 80 dB SPL) 24h a day for two weeks. The noise level varied less than 2 dB within the space of the cage.

3.5. Measurement of lateral wall stiffness

Stiffness of the lateral wall was measured by the glass micropipette aspiration technique combined with a pseudocolor image analysis method. Glass micropipettes were pulled from Clark EC15TF capillaries with an inner diameter of 2.8 μm . Pipettes were filled with extracellular solution and connected to the calibrated water column controlled pressure application apparatus through a polyethylene plastic tube. The stiffness parameter (S_p) was determined in the midlateral region of OHCs and was calculated from the length of the aspirated lateral wall segment into the suction pipette at the end of a 30 s-period of 6H₂Ocm negative pressure. All measurements were carried out within 40 min after the death of the animal.

3.6. Data analysis for stiffness measurements

Statistical comparisons were made using Student's t-test, and when appropriate, paired t-test at $p=0.05$. For all experiments, the standard error of the mean (SE) is reported.

3.7. Electrophysiology

Whole-cell patch clamp measurements were carried out using Axopatch-200 and Axopatch-200A amplifiers connected to personal computers using Axon Instruments TL-1-125 and Digidata 1200 computer interfaces, respectively. For data acquisition and analysis the pClamp8 software package (Axon Instruments Inc., Foster City, CA) was used. Standard whole-cell patch-clamp techniques were used, as described (Hamill et al. 1981). Cells were held at -70 mV. Data acquisition was done at 2 kHz sampling frequency, filtering was set to 1 kHz. Pipettes were pulled from GC150F-15 borosilicate glass capillaries (Clark Biomedical Instruments, UK) in five stages and fire-polished to give electrodes of 4-6 M Ω resistance in the bath. The external solution contained (mM): NaCl 140, CaCl₂ 2, MgCl₂ 1, KCl 5.4, glucose 10, and HEPES 10 (pH 7.4). The pipette filling solution contained (mM): KCl 140, MgCl₂ 1, EGTA 10, and HEPES 10 (pH 7.3). Series resistance of the cells was generally between 12 and 25 M Ω . When a whole-cell current in excess of 1 nA was detected we applied series resistance compensation up to 50-70% to reduce voltage errors.

3.8. Test substances for patch-clamp recordings

Tetraethylammonium-chloride (TEA-Cl, Sigma Aldrich) solutions were prepared by substituting NaCl for equimolar concentrations of TEA-Cl. Charybdotoxin (ChTx) and Margatoxin (MgTx) were obtained from Alomone Labs (Jerusalem, Israel), Pi1 toxin was a generous gift of Lourival D. Possani (Institute of Biotechnology, UNAM, Mexico). Bath perfusion with different test solutions was achieved using a gravity-flow perfusion setup with 6 or 8 input lines and a PE10 polyethylene tube output tip with flanged aperture to reduce the turbulence of the flow. The solutions were applied in an alternating sequence of control and test solutions, unless stated otherwise. Switching was done either manually or via electromagnetic valves. Excess fluid was removed continuously from the bath.

3.9. Patch-clamp data analysis

Prior to analysis whole cell current traces were corrected for ohmic leak and digitally filtered (3 point boxcar smoothing). Non-linear least squares fits were done using the Marquardt- Levenberg algorithm. Fits were evaluated visually as well as by the residuals and the sum of squared differences between the measured and calculated data points. Statistical comparisons were made using Student's t-test at $p=0.05$. For all experiments, the standard error of the mean (SE) is reported.

4. RESULTS

4.1. Calcium transients on OHCs

Resting intracellular calcium concentration in the normal OHCs was found to be 120 ± 4 nM (mean $\pm$ SE, $n=35$), and the cells represented, in this respect, a homogenous population. When $180 \mu\text{M}$ ATP was added to the extracellular solution for 30 seconds, the rise in intracellular calcium concentration could be separated into two classes based on their kinetic behavior. In a subpopulation of cells ($n=14$) a fast response was observed. The intracellular calcium concentration rose by 186 ± 36 nM (mean $\pm$ SE), with a latency of 5.9 ± 1.0 s, and returned to the resting level. The response was repeatable. After successive additions of ATP, responses with identical amplitude and kinetics could be evoked on these cells. In another group of cells ($n=15$) the intracellular $[\text{Ca}^{2+}]_i$ increased by only 74 ± 10 nM with a latency time of 12.3 ± 1.9 s. The increase of the intracellular calcium level was thus lower, it developed slower, and, furthermore, the $[\text{Ca}^{2+}]_i$ did not return to the resting value. Note also that the ATP-induced $[\text{Ca}^{2+}]_i$ transient could not be evoked again with a second addition of ATP.

The presence of these two types of responses suggested the existence of more than one receptor subtype in the membrane of the OHCs. Since subsequent experiments revealed the presence of P_{2Y} receptors on these cells, we tested the effect of UTP. Calcium transients evoked by $180 \mu\text{M}$ UTP had smaller amplitude (48 ± 11 nM, $n=5$) and slower rising phase as compared to those evoked by ATP. Furthermore, 58% of cells that responded to ATP did not respond to UTP. The kinetics of the UTP-evoked signal resembled, to some extent the slow-type signal seen with ATP application. Pre-incubation of the cells with $300 \mu\text{M}$ suramin, an antagonist of the purinergic receptors, greatly suppressed (to 26 ± 10 nM, $n=9$) the response to subsequent addition of ATP.

4.2. Purinoreceptor subtypes on OHCs

Four P_{2X} and three P_{2Y} receptor subtypes were detectable in the membrane of the guinea pig outer hair cell. Using antibodies against P_{2X1} , P_{2X2} , P_{2X4} , P_{2X7} receptors, we found intensive labeling with P_{2X1} , P_{2X2} , P_{2X4} , whereas only a weak signal was detected with the antibody directed to the P_{2X7} receptor. The distribution of the receptor subtypes along the outer hair cell was also different. While the distribution of P_{2X1} and P_{2X4} receptors was homogenous, the P_{2X7} receptor could only be observed weakly at the basal pole while the P_{2X2}

mostly at the apical part, close to the cuticular plate. Similar studies revealed strong immunolabeling of all three P_{2Y} receptors studied. The labeling was homogenous with P_{2Y1} , while it was only detectable at the apical part of the cell with P_{2Y2} , and at the basal pole of the cell with P_{2Y4} .

4.3. Calcium transients on noise treated OHCs

Resting intracellular calcium concentration of the non treated OHCs was found to be 133 ± 12 nM ($n=14$). After the addition of $180 \mu\text{M}$ ATP for 30 s to the cells, the intracellular calcium concentration rose by 266 ± 65 nM with a latency of 5.9 ± 1.0 s. Since there was no statistical difference between the data of the apical and basal cells in the control cell population, the two groups were mixed and used together as a control group.

After the noise exposure some of the cells showed degenerative signs and abnormal cell shape, they were more sensitive to injury and did not respond to ATP. Those cells fitting the morphological criteria of a healthy cell showed the resting intracellular calcium concentration was significantly higher. We also found a slight difference between the cells which originated from the apical and the basal turns. The resting calcium concentration in the noise exposed cells of the apical two turns was 181 ± 24 nM ($n=11$) whereas in the cells of the basal turn it was even higher, 219 ± 29 nM ($n=8$). In line with these observations, the response of the cells to $180 \mu\text{M}$ ATP for 30 s, was smaller with a longer latency time, and we again found a slight difference between the two cell populations in this respect. The intracellular calcium concentration rose by only 60 ± 22 nM with a latency of 8.4 ± 2.2 s ($n=11$) in the apical turns, whereas by 44 ± 10 nM with a latency of 9.9 ± 1.1 s ($n=8$) in the basal turn.

4.4. Purinoreceptor subtypes on OHCs after noise exposure

According to the previous morphological and functional experiments, namely that the basal part of the cochlea is more susceptible to the effect of noise, cells from the basal and the apical parts of the cochlea were separated in the subsequent experiments. The expression level of P_{2X1} , P_{2X2} , P_{2X4} , P_{2X7} and P_{2Y1} , P_{2Y4} receptor subtypes was lower, while that of the P_{2Y2} receptor subtype did not change in the noise-exposed animals. We did not find any significant difference in respect of the receptor expression between the cells isolated from the apical and basal parts of the cochlea. The expression of all purinoceptors was homogenous in the noise-exposed group.

4.5. Effect of noise exposure on the stiffness of the lateral wall of outer hair cell from different cochlear locations

Mechanical stimuli initialize the regulatory stiffness response, an intrinsic regulatory system of OHCs. The regulatory stiffness response increases the OHC lateral wall stiffness which may play a protective role against long term noise stimulus.

Under these experimental conditions, no significant difference was found between the Sp of cochleobasal and cochleoapical cells of control animals. However, the influence of noise exposure on the stiffness of the lateral wall of the OHCs was not dependent on the localizations of the cells along the cochlea.

4.6. Investigation of the voltage sensitive potassium currents in isolated Deities cells of the guinea pig cochlea

The cells were held at -70 mV and depolarized to different test potentials ranging from -70 mV to $+70$ mV for 10 s every 60 s. Currents could be elicited at test potentials more positive than -30 mV, the activation kinetics of the currents became faster and the peak currents increased with increasing depolarization of the membrane. These indicate that voltage-gated channels are responsible for the recorded currents. Conventional tail current measurements were used to determine the reversal potential of the whole-cell current. The cell was held at -70 mV and depolarized to $+70$ mV for 50 ms followed by a step to different test potentials ranging from -120 mV to -20 mV in 10 mV increments in every 30 s. The value obtained for the reversal potential was -69.3 ± 2.4 mV ($n=5$), which is close to the calculated reversal potential of K^+ ions calculated from the Nernst equation using the K^+ composition of the pipette and bath solutions (-83.5 mV). Based on these data we concluded that this current is highly K^+ selective. K^+ current of a total of 77 Deities cells were recorded and a large cell-to-cell variability was found in the observed current amplitudes (Figure 1b), the median of the peak currents at $+50$ mV test potential was 3.66 nA, however, extreme values of 0.7 nA and 13.2 nA were also observed.

4.7. Separation of K⁺ current components based on the inactivation kinetics

The decay of the whole-cell currents during sustained (10-s-long) depolarization is slow and at test potentials more positive than 0 mV the decay is clearly biphasic containing a faster and a slower component. The biphasic decay of the currents may also reflect the existence of two K⁺ channel types in Deiters cells.

Current traces recorded in Deiters cells at +50 mV test potential. The biphasic decay of the current most likely reflects the inactivation of the K⁺ channels, and inactivation time constants τ_1 and τ_2 were used to describe the inactivation rate of the slow and the fast components of the current decay, respectively. The inactivation time constants were obtained by fitting the decaying part of the currents using the sum of two exponential terms: $I = A_1 \times (e^{-t/\tau_1}) + A_2 \times (e^{-t/\tau_2}) + C$, where A_1 and A_2 are the amplitudes of the slower and faster components, respectively, C is the non-inactivating current component.

The inactivation time constants showed little cell-to-cell variability, the values of τ_1 and τ_2 were 2735 ± 90 ms ($n=77$), and 160 ± 14 ms ($n=72$), respectively. On the contrary, the relative weight of the slowly inactivating current component showed a large scatter, the ratio of $A_1/(A_1 + A_2)$ was between 0.4 and 0.9. We also found 5 cells which showed K⁺ currents with monophasic inactivation kinetics. In these cells, the decay of the current could be fitted by a single exponential function. The inactivation time constant characterizing the exponential decay ($\tau=3008 \pm 188$ ms) was similar to the slower inactivation time constant (τ_1) measured in cells showing biphasic inactivation kinetics.

The appearance of the biphasic inactivation kinetics at large depolarization indicated that the voltage-dependence of steady-state activation for the two current components may be different, and this scenario was studied in detail. The peak current-voltage (I-V) relationship of the K⁺ current was measured from traces obtained using 100 ms-long voltage steps to test potentials ranging from -120 mV to +100 mV in 10 mV steps from a holding potential of -70 mV. From the values of the peak currents the conductance at each test potential was calculated according to the following equation: $G_K = I_K / (E_m - E_K)$ where G_K is the whole-cell conductance of the membrane for K⁺, I_K is the peak current, E_m is the actual membrane potential, E_K is the reversal potential for K⁺. The G_K values were normalized to the maximal one to get G_{norm} and plotted as a function of the test potential. To determine the characteristic values of the voltage-dependence of steady-state activation of each current component separately, the G-V relationships were fit with a constrained sum of two Boltzmann functions. The applied constrain was the relative weight of the two current components determined from

the analysis of the inactivation kinetics of the currents at +50 mV. The final form of the equation used to fit the G-V relationships was:

$$G_{\text{norm}} = \frac{A_1 / (A_1 + A_2)}{1 + e^{-\frac{V - V_{1/2,1}}{k_1}}} + \frac{A_2 / (A_1 + A_2)}{1 + e^{-\frac{V - V_{1/2,2}}{k_2}}},$$

where $V_{1/2,1}$ and k_1 are the midpoints and the slope of the G-V relationship for the slowly inactivating component, respectively, whereas index 2 refers to the same parameters obtained for the faster component. The resulting values were $V_{1/2,1} = -1.42 \pm 2.68$ mV, $k_1 = 11.93 \pm 1.15$ mV, $V_{1/2,2} = 12.48 \pm 3.85$ mV, $k_2 = 15.2 \pm 3.6$ (n=12), indicating a ~14 mV separation of the G-V relationships.

An additional measure of $V_{1/2}$ and k values came from fitting the inactivation kinetics of the currents and determination of the amplitudes (A_1 and A_2) of the two current components at different test potentials. From the current amplitudes the G-V relationships were constructed for the slow and the fast component *separately* using the same approach as described above. Fitting of single Boltzmann functions to the G-V relationships resulted in $V_{1/2,1} = -3.38 \pm 2.84$ mV, $k_1 = 12.24 \pm 1.81$ mV and $V_{1/2,2} = 9.1 \pm 1.06$ mV, $k_2 = 9.68 \pm 2.73$ (n=3).

Amplitudes (A_1 and A_2) determined from fitting the inactivation kinetics of the currents was also used to construct the voltage-dependence of steady-state inactivation curves for the two current components, separately. The holding potential was -70 mV, and pairs of 10-s-long depolarizing pulses were applied to +70 mV separated by 150-s-long holding periods ranging from -65 to 0 mV in 5 mV increments. The inactivation kinetics of the currents were fit using a sum of two exponential terms, and the ratios of the amplitudes of the current components evoked from -70 mV holding and from a more depolarized holding (h) were calculated, i.e., the $A_{1,h}/A_{1,-70}$ and the $A_{2,h}/A_{2,-70}$ ratios indicate the fraction of non-inactivated channels for the slow and the fast component of the current, respectively, at membrane potential h . Due to the extremely long duration of the protocol 3-4 data points per cell were collected, and data obtained in different cells were averaged at each test potential to construct the voltage-dependence of steady-state inactivation curves shown in Fig. 2 e and f. Curves were fitted using a single Boltzmann-function, the resulting values were $V_{1/2,i,1} = -33.7$ mV, $k_{i,1} = -5.7$ mV, $V_{1/2,i,2} = -28.3$ mV, $k_{i,2} = -4.2$ mV, indicating a small ~5 mV separation of the voltage dependence of steady-state inactivation. These results in conjunction with the voltage-dependence of steady-state activation argue that the voltage-dependent gating of the two current components is very similar.

4.8. Pharmacological separation of the current components

Although the analysis of the inactivation kinetics of the currents already indicated the presence of two types of K^+ channels in Deiters cells we attempted to clarify it by pharmacological separation of the currents using K^+ channel blockers. Our expectation was that a blocker having different affinities for the two channel types will result in the decrease of the amplitude accompanied by a change in the decay of the whole cell currents due to the specific removal of one component.

We examined the effect of a non-peptide blocker, tetraethylammonium (TEA) and peptide blockers (Margatoxin, Charybdotoxin, Pandius Imperator scorpion toxin) of K^+ channels on the amplitude of the two current components and the change in the kinetics of the current decay. During these experiments we used +50 mV steps for 10 seconds from a holding potential of -70 mV. By fitting the decay of the current traces using a sum of two exponential terms we determined the remaining fractions (RF) of the two current components from the $RF(A_1)=A_1/A_{1,0}$ and $RF(A_2)=A_2/A_{2,0}$ ratios, where A_x and $A_{x,0}$ are the amplitudes in the presence of the blocker and in control conditions, respectively.

In the presence of 1 mM TEA, and the blocked current obtained by taking the difference of the control trace (control) and the one recorded in the presence of TEA. The overall RF at 1mM TEA concentration was 0.808 ± 0.032 (n=3), the remaining fraction of the slow current component was $RF(A_1)= 0.740 \pm 0.045$, (n=3), whereas that of fast component was $RF(A_2)=0.953 \pm 0.032$ (n=3). At higher TEA concentrations both of the current components were inhibited significantly. The dissociation constants obtained for the slow and the fast current components were 2.63 mM and 6.70 mM, respectively.

Margatoxin (MgTx) and Pandius Imperator scorpion toxin (PI1) blocked almost equally the two components of the outward currents (data not shown), while Charybdotoxin (ChTx) blocked the two current components by different affinities. Application of 10 nM ChTx substantially reduced the fast component of the current decay. The blocked current was obtained by taking the difference of the control trace (control) and the one recorded in the presence of the toxin (10 nM ChTx). The figure clearly shows that the blocked current is dominated by the more rapidly inactivating current component, although, some of the slower component was also blocked. The RFs of the individual current components in the presence of 10 nM ChTx were 0.722 ± 0.057 and 0.290 ± 0.057 for the slow and the fast component (n=5), respectively.

4.9. Expression of K⁺ currents in morphologically different Deiters cells.

The cell isolation procedure yielded isolated Deiters cells and Deiters cells attached to outer hair cells (OHCs). The amplitude of outward K⁺ current at +50 mV was significantly higher in Deiters cells connected to OHCs than in isolated Deiters cells, the average peak currents were 5417 ± 541 pA (n=40) and 3527 ± 410 pA (n=37) in OHC-connected and isolated Deiters cells, respectively (p=0.0068). Since gap junctions can connect OHCs and Deiters cells, the measured whole-cell currents could be the sum of currents of the Deiters cell and the OHC connected to the Deiters cell. In this case the measured cell capacitance would be higher than in isolated Deiters cells. The measured cell capacitance was 35.91 ± 2.02 pF (n=29) for Deiters cells attached to OHCs and 35.25 ± 2.35 pF (n=25) for isolated Deiters cells, these values were not different significantly (p=0.83).

We also found a morphological diversity among the Deiters cells in our preparations. Those cells, which attached to shorter OHCs, had larger cell bodies (“corpulent cells”) whereas others, which attached to longer OHCs, had “lanky” cell bodies. Qualitatively, the longer the length of the OHCs was, the greater the length of the phalangeal process of Deiters cells attached to the given OHC was. Based on these qualitative differences, the cells were separated into two morphological groups: Deiters cells with a corpulent body and short phalangeal process whereas the other group contained Deiters cells with a “lanky” body and long phalangeal process.

There the peak currents measured at +70 mV were 3697 ± 497 pA (n=29) for the corpulent cells and 5207 ± 573 (n=36) for the lanky cells. There was no significant difference in the cell membrane capacitance of these two groups of cells [lanky cells: 37.36 ± 2.64 pF (n=19); corpulent cells: 33.13 ± 2.11 (n=25), p=0.0136], whereas, the difference in the current amplitudes normalized to the cell capacitance was statistically significant. The current density was significant higher in Deiters cells with lanky cell body [188.11 ± 23.14 pA/pF (n=19)], than in corpulent cells [114.23 ± 17.23 pA/pF (n=25)] (p=0.012). The shape of the currents observed at +50 mV during 10-second-long pulses also differed between the two groups, the overall inactivation rate was slower in the corpulent cells. The inactivation time constants were the followings: $\tau_1 = 2718 \pm 117$ ms (n=27), $\tau_2 = 190 \pm 28$ ms (n=25) for the cells with corpulent bodies and $\tau_1 = 2669 \pm 120$ ms (n=36), $\tau_2 = 136 \pm 17$ ms (n=35) for the cells with lanky bodies. There was no significant difference either in case of τ_1 values (p=0.777), or in the case of τ_2 values (p=0.088). The slower inactivation of corpulent cells originates from the

larger weight of the slowly inactivating component of these cells compared to the lanky cells. The weight of the slow component [$A_1/(A_1 + A_2)$] was 0.736 ± 0.033 for corpulent cells ($n=27$) and 0.522 ± 0.023 for lanky cells ($n=36$). In agreement with this latter result, the analysis of the amplitudes of the two current components normalized to the cell capacitance showed that the normalized amplitude of the slower current component is the same in lanky and corpulent cells [lanky: 85.65 ± 11.98 pA/pF ($n=19$); corpulent: 69.88 ± 10.05 pA/pF ($n=23$), $p=0.316$], whereas the amplitude of the faster current component is significantly greater in cells having lanky cell body [lanky: 74.61 ± 10.07 pA/pF ($n=19$); corpulent: 25.83 ± 6.20 pA/pF ($n=23$) ($p=0.0002$)].

5. DISCUSSION

5.1. Examination of ATP induced intracellular Ca^{2+} changes, and expression of purinergic receptor subtypes in the outer hair cells of the guinea pig cochlea

According to previous experiments the P_2 receptors have a wide distribution in the inner ear, but only $\text{P}_{2\text{X}2}$ and $\text{P}_{2\text{X}7}$ were shown to be localized in the membrane of the isolated outer hair cell. To our knowledge it has not yet been described whether these receptors are homogeneously distributed or are localized to specific areas within the cell. Interestingly, Mammano presented evidence that the ATP evoked changes in $[\text{Ca}^{2+}]_i$ are first detected at the apical end. Together with the observations in this study these measurements suggest an important role for the $\text{P}_{2\text{X}2}$ or $\text{P}_{2\text{Y}2}$ receptors in the initiation of the response. This is also in line with previous studies localizing $\text{P}_{2\text{X}2}$ receptors in the membrane of OHCs.

When 180 μM ATP (30 s) was added to the cells a transient, fast $[\text{Ca}^{2+}]_i$ response with high amplitude was seen in a subpopulation of OHCs. This is characteristic of the $\text{P}_{2\text{X}1}$ receptor subtype. The sensitivity of this receptor for ATP is the highest within the $\text{P}_{2\text{X}}$ family with an EC_{50} of 1 μM rendering this receptor a possible target for the low ATP concentration present in the endo- and perilymph. However, this receptor subtype is known of its fast and prolonged desensitization in contrast with the repeatable response observed in this study. Taken together, these data suggest that the calcium transient seen on these cells is likely to result from interplay between several $\text{P}_{2\text{X}}$ receptor subtypes.

In another group of cells the response was smaller, the development of the response was slower and the intracellular calcium level did not return to the resting level. This response may be due to the opening of other ($\text{P}_{2\text{X}2}$, $\text{P}_{2\text{X}4}$, $\text{P}_{2\text{X}7}$, $\text{P}_{2\text{Y}}$) receptor subtypes which have higher EC_{50} for ATP (10-100 μM). When those receptors, especially the $\text{P}_{2\text{X}7}$ are open, the intracellular calcium level does not return to the resting level which can lead to the death of the cell. On the other hand, the presence of an UTP-evoked calcium signal together with the positive immunolabelling for $\text{P}_{2\text{Y}}$ receptor subtypes, strongly argue in favor of a metabotropic signaling pathway, too. It is interesting to note here that some cells did not respond to UTP while they displayed normal sensitivity against ATP. This observation, together with the fact that other cells responded to both UTP and ATP, suggests that OHCs might represent a heterogeneous group in respect to their purinoreceptor expression pattern, too. Namely, a subpopulation of cells might only express $\text{P}_{2\text{X}}$, while another sub-population both $\text{P}_{2\text{X}}$ and $\text{P}_{2\text{Y}}$ receptors.

According to these observations purinoceptors are widely present in both the membrane and the intracellular compartment of the outer hair cells with a different distribution pattern, and ATP may play an important role in the modulation of the hearing mechanism through regulation of $[Ca^{2+}]_i$ and subsequently, the force feedback of the basilar membrane.

5.2. Effect of moderate level noise on the resting Ca^{2+} level, changes in the ATP induced Ca^{2+} transients and expression changes in the purinergic receptor subtypes in the membrane of the outer hair cells.

According to previous experiments, moderate noise exposure (1 kHz, 81 dB SPL, 24 days) did not cause damage of the organ of Corti and OHC loss in guinea pigs, which was proved by morphological and functional/audiological experiments. Contrary to this observation low-level sound exposure (65 dB SPL narrow-band noise for 10-11 days) can change cochlear function.

Some authors explain the noise induced functional changes with an increase in the intracellular calcium level after acoustic overstimulation. Noise as a mechanical stimulus depolarize the resting membrane potential. This depolarization activates voltage sensitive calcium channels which permit a continuous calcium influx. In our experiments, we also found an increase in the resting intracellular calcium concentration in the OHCs, which was similar to the former investigation of the effect of high level (130 dB SPL) noise exposure. Based on the previous morphological and functional experiments, namely that the basal part of the cochlea is more susceptible to the effect of noise, we made two different preparations. Among the noise-exposed animals not only was the measured resting intracellular calcium concentration in the OHCs higher, but we also found a slight difference between the cells which originated from the apical as compared to those from the basal turns. Namely, the calcium level was slightly higher in the cells of the basal turn (219 nM) than in the apical turns (181 nM). This higher resting intracellular concentration may explain the swelling or loss of OHCs, which could be observed mainly in the basal 20% of the organ of Corti in previous experiments.

Noise exposure can modify the response of OHCs to different neurotransmitters, such as to ATP measured by former patch-clamp experiments. In line with this finding, we observed, that the change in the intracellular calcium level after the addition of 180 μ M ATP for 30 s was lower and had a longer latency time in noise-exposed animals. We also found a slight difference between cells originating from different parts of the cochlea. The intracellular

calcium level rose by 60 nM in cells of the apical turns, and only by 44 nM in the cells from the basal turn, significantly less than the response of the non-exposed animals (the amplitude of the response of the control animals to 180 μ M ATP was 266 nM with a latency of 5.9 s). This observation is clearly in line with the difference found in the intracellular calcium levels.

The constant efferent stimulation of the OHCs and the subsequent higher level of the intracellular calcium concentration may cause down-regulation of the membrane purinoreceptors, and this down-regulation may be in the background of the delayed and smaller response of the cells to ATP. Using polyclonal antibodies against several subtypes of purinoceptors, we found lower intensities with P_{2X1}, P_{2X2}, P_{2X4}, P_{2X7} and P_{2Y1}, P_{2Y4} subtypes, while the intensity of P_{2Y2} receptor subtype did not decrease in the noise-exposed as compared to the non-treated animals. Due to the difficulty of quantifying immunofluorescence we could not resolve significant differences in the expression intensity between the cells from the apical and basal turns of the cochlea.

An increase in the intracellular calcium concentration is assumed to cause a decrease of the stiffness of OHCs (axial and transversal). The decreased stiffness of OHC increases the vulnerability of the cells against mechanical insults. The regulatory stiffness response can not only compensate the decreased stiffness due to the increased intracellular calcium level but it can increase this comparing to the normal level. In line with this expectation and in parallel with the increase of the resting intracellular calcium concentration, we found an increase of the stiffness of the lateral wall of the OHCs. One of the possible explanation of the phenomenon is the recently described Ca²⁺ dependent, stretch-induced regulation of the lateral wall stiffness (regulatory stiffness response), which is controlled by efferent neurotransmitters. This response is processed by the cytoskeleton and causes an increase in the lateral wall stiffness of the OHCs. Loud sounds of long duration vibrate the basilar membrane with greater amplitudes, which might result in a greater stretch of the lateral wall. This may result in a permanent mechanical stimulus, which causes a persistent increase in lateral wall stiffness. An additional explanation for our results could be a metabolic modification in the cell membrane or subcortical cytoskeleton which may also modify the stiffness of the lateral wall.

Change in the intracellular calcium level and the operation of stretch-gated ion channels play an important role in the process of the regulatory stiffness response. New calcium signal is needed to elicit this response. The long term increase in the resting intracellular calcium level causes a consecutive decrease in the amplitude of the calcium

signals. This mechanism may be the cause that no significant difference was found between the Sp of cochleobasal and cochleoapical cells. of control animals

In conclusion, noise-induced changes in the intracellular calcium homeostasis and subsequently in the calcium dependent regulatory mechanisms, may lead to the modification of the OHC lateral wall stiffness. The changes in the micromechanical properties of the OHCs may interfere with the peripheral auditory processing leading to the reduction of the efficacy of the cochlear amplifier.

5.3. Examination of voltage sensitive potassium currents of the Deiters cells with different morphology

The putative role of the Deiters cells in the cochlear micromechanics, as well as, in the electrolyte homeostasis of the inner ear is still obscure. Former experiments revealed that in the Deiters cells more than one type of K^+ channels might be responsible for whole-cell potassium current.

During 10-second-long depolarizing pulses the outward current had a biphasic inactivation. The biphasic inactivation process may represent expression of two voltage-gated K^+ channels with different rates of inactivation. The other possibility is that one channel is responsible for this current but the inactivation process has two distinctly different time constants, which appears as biphasic inactivation, as it is the case for several voltage-gated K^+ channels. To distinguish between these scenarios we characterized the biophysical and pharmacological properties of the K^+ current.

A number of evidence argues for the existence of two different K^+ channel types: First, five cells showed monophasic inactivation kinetics, and the time constant characterizing the decay was similar to the slower component of biphasic decays. The lack of the fast component of the decay argues for the expression of one type of K^+ channels in these cells. Second, ChTx and TEA reduced the amplitude of the more rapidly and slowly inactivating current components differentially: in the presence of 10 nM ChTx the blocked current is dominated by the more rapidly inactivating current component whereas in the presence of 1 mM TEA predominantly the slow component was blocked. Third, the more rapidly inactivating current component appeared at large depolarizations as if the voltage-dependence of steady-state activation was shifted towards depolarized potentials for these channels as compared to those responsible for the slow component of the decay. Fourth, the constrained fits of the G-V relationships using the sum of two Boltzmann functions resulted in different

$V_{1/2}$ values for the two components, the $V_{1/2}$ of the more rapidly inactivating component being shifted toward depolarized potentials by ~ 14 mV. A similar shift in the $V_{1/2}$ was obtained when the voltage-dependence of steady-state activation was constructed from the amplitudes of the individual current components (A_1 , A_2) obtained from the fitting of the biphasic inactivation kinetics using a sum of two exponential terms, which indicates the reliability of the reported values. In addition, the voltage dependence of steady-state inactivation was also different for the two current components, the $V_{1/2,i}$ of the more rapidly inactivating component being shifted toward depolarized potentials by ~ 5 mV.

The biophysics and the limited pharmacology did not allow the exact molecular characterization of the K^+ channel types being responsible for the whole-cell current. Considering the ChTx sensitivity of the more rapidly inactivating current, its K_d for TEA (6.7 mM) and its inactivation kinetics ($\tau_2 \sim 160$ ms) the most likely candidate channel being responsible for this current component is Kv1.3. Other ChTx sensitive channels are excluded based on the resistance to TEA block (Kv1.2) or by the difference in the gating mechanism (BK channel). The molecular identity of the channels giving rise to the slowly inactivating current component remains to be elucidated.

The examined Deiters cell population was not homogenous. We studied both isolated cells and cells connected to OHCs and we found a difference in the current amplitude between the two groups. Two possibilities were assumed to explain this observation. The first, considering the sum of the K^+ currents of the two cells (OHCs and Deiters cells) connected via gap junctions, was rejected because the cell capacitance of isolated Deiters cells was the same as in those in morphological contact with OHCs. Therefore, higher channel expression (increase in the number of channels) or up-regulation of the K^+ conductance may cause the higher current amplitude on Deiters cells connected to OHCs.

It was described earlier that the morphology and cell shape of the Deiters cells depends on the length of the attached OHCs and consequently, their location in the organ of Corti. Deiters cells increase in size from base to apex, and from the first row towards the more lateral third row. Deiters cells were separated into two groups in this study: those with corpulent body, which were attached to shorter OHCs, and others with lanky cell body supporting longer OHCs. Our data showed that lanky and corpulent cells have similar K^+ channels as the inactivation time constants characterizing the biphasic current decays are statistically not different in the two groups. However, lanky cells have a larger current amplitude which is attributed to an increase in the magnitude of current component obeying faster inactivation kinetics. Although this clearly indicates that the relative proportion of different K^+ channels

depends on the morphological characteristics of Deiters cells, the functional significance of this feature is not known.

The contribution of Deiters cells to cochlear micromechanics has been raised by several authors. These cells receive afferent innervation, feature voltage sensitive K^+ conductances and perform active stalk movement. The potential link between K^+ channel expression of Deiters cells and modulation of active cochlear micromechanics is intriguing, but remains to be elucidated. The outward rectifying voltage-gated K^+ channels characterized in this study are probably also involved in the restoration of the membrane potential after depolarization insults, e.g., following P_{2X} receptor activation. One of the physiologically important functions of supporting cells, such as Deiters cells, is the regulation of K^+ recycling in the inner ear. Release of K^+ by OHCs following mechanical stimulation is followed by siphoning of K^+ into Deiters cells mediated by the K^+-Cl^- co-transporter *Kcc4*, and subsequently, K^+ enters into the gap junction pathway. The diverse membrane potential dependence of the gap junction channels in Deiters and other supporting cells could result in asymmetric current flow between the cells and influence K^+ passage. This membrane potential-dependent mechanism might effectively be influenced by K^+ channels and the dominance of distinct K^+ channel types in morphologically different Deiters cells described in this study.

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