ATP-evoked intracellular Ca^{2+} signaling of different supporting cells in the hearing mouse hemicochlea

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Abbreviations:
AM, acetoxymethyl; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CICR, calcium-induced calcium release; CCD, charge-coupled device; CPA, cyclopiazonic acid; EC_{50}, half maximal effective concentration; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; ER, endoplasmic reticulum; RT-PCR, real-time polymerase chain reaction; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

Abstract

Hearing and its protection is regulated by ATP-evoked Ca^{2+} signaling in the supporting cells of the organ of Corti, however, the unique anatomy of the cochlea hampers observing these mechanisms. For the first time, we have performed functional ratiometric Ca^{2+} imaging (fura-2) in three different supporting cell types in the hemicochlea preparation of hearing mice to measure both P2X and P2Y receptor-mediated Ca^{2+} signaling in pillar, Deiters' and Hensen's cells. Their resting $[Ca^{2+}]_i$ was determined and compared in the same type of preparation. ATP evoked reversible, repeatable and dose-dependent Ca^{2+} transients in all three cell types, showing also desensitization. Inhibiting the Ca^{2+} signaling of the ionotropic P2X (by omission of extracellular Ca^{2+}) and metabotropic P2Y receptors (by depleting the intracellular Ca^{2+} stores) revealed the involvement of both receptor types. Detection of the mRNAs of $P2X_{2,3,4,6,7}$ and $P2Y_{1,2,6,12,14}$ receptors by RT-PCR supported this finding. However, while the sum of the extra- and intracellular Ca^{2+}-dependent components of the response was about equal with the control ATP response (linear additivity) in pillar cells, the summation of the respective P2X and P2Y receptor-mediated components in Deiters' and Hensen's cells showed supralinearity. Calcium-induced calcium release might explain this synergistic interaction. The more pronounced Ca^{2+} leak from the endoplasmic reticulum in Deiters' and Hensen's cells, unmasked by cyclopiazonic acid, may also suggests the higher activity of the internal stores in Ca^{2+} signaling in these cells. Differences in Ca^{2+} homeostasis and ATP-induced Ca^{2+} signaling might reflect the distinct roles these cells play in cochlear function and pathophysiology.

Keywords: hemicochlea, Ca^{2+} imaging, ATP, pillar cells, Deiters' cells, Hensen's cells

Introduction

Hair cells, the sensory receptors in the organ of Corti are surrounded by a glia-like network of supporting cells including pillar, Deiters' and Hensen's cells. After a long inferior role, supporting cells are emerging as central players in the inner ear [1]. They are supposed to help maintaining cochlear homeostasis and also play an important active role in normal functions and pathological processes in hearing like cochlear amplification [2, 3] and protection against excessive noise exposure [4]. However, the specific physiological and pathophysiological role of the different supporting cells and their regulation have not been well explored.

ATP signaling has a central role in sensory transduction. By stimulating its seven ionotropic P2X ($P2X_{1-7}$) and eight metabotropic P2Y ($P2Y_{1-2}$, $P2Y_4$, $P2Y_6$ and $P2Y_{11-14}$) receptors, it regulates diverse functions in auditory physiology and pathophysiology [5, 6]. Although it is not investigated systematically based on species, age and receptor subtype, there are several lines of evidence showing the presence of P2X and P2Y receptors in the cochlea, including supporting cells of the organ of Corti (cf. Housley et al., 2009). Intracellular Ca^{2+} seems to be the main second messenger in ATP-mediated signaling [7–9].

ATP is widely distributed in the inner ear [5]. It can be released to the endolymph by the stria vascularis [10, 11] but cells of the organ of Corti also use ATP as a paracrine mediator [12, 13]. Both hemichannel-mediated [13] and Ca^{2+}-dependent vesicular release were suggested [12] but ATP can also escape from injured hair cells and transfer the information of damage to the surrounding supporting cells [4, 14].

Extracellular ATP controls the intercellular Ca^{2+} waves, which travel through supporting cells and are suggested to take an important part in the regulation of the K^+ recycling and repair mechanism in noise trauma (Mistrik and Ashmore, 2009; Zhu and Zhao,

2010) (Piazza et al., 2007). Altering the function of this ATP-mediated connexin-based network of the supporting cells results in hearing impairment [2, 19, 20].

The purinergic transmitter ATP can modify hearing sensitivity through other actions on the supporting cells, as well. ATP may influence active cochlear micromechanics and the cochlear amplifier via inducing the movement of the stalks, shown on isolated Deiters' cells [21]. Increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) also caused the immediate movement of the head of the Deiters' cell's phallangeal process, as it was shown by photorelease of caged-Ca^{2+} [22].

Although the phenomenon of ATP-evoked intracellular Ca^{2+} response have been shown in different types of supporting cells, including Deiters', Hensen's and pillar cells, these studies did not explore the precise role and interplay of the P2X and P2Y receptors and were largely performed on isolated cells [22–26] or in neonatal tissue [4, 18, 27]. The sparse functional Ca^{2+} imaging studies on in situ young adult preparations missed to show any ATP-evoked Ca^{2+} transient in pillar, Deiters' or Hensen's cells [28] or were focusing solely on one of the cell types [29, 30].

A study which investigates both P2X and P2Y receptor-mediated purinergic signaling in all three types of cells in the same preparation was missing. Functional Ca^{2+} imaging measurements in supporting cells of the organ of Corti were performed in the *in situ* hemicochlea preparation from hearing mice for the first time. The hemicochlea technique [31–34] provides a radial perspective for observation of the cochlear material that retains the delicate cytoarchitecture of the organ of Corti and ensures an advantage over experiments on isolated cochlear cells or on tissue prepared from mice with immature hearing. Here we measured and compared the basal $[Ca^{2+}]_i$, the ATP-evoked Ca^{2+} transients and the involvement of the ionotropic, extracellular Ca^{2+}-dependent P2X and the metabotropic, intracellular store-dependent P2Y signaling of the three supporting cell types, in the same

experimental model. The results suggested the role of both P2X and P2Y receptor-mediated ATP signaling in all three cell types but a higher leak of Ca^{2+} from the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-dependent Ca^{2+} stores and a possible involvement of calcium-induced calcium release (CICR) in Deiters' and Hensen's cells, compared to the pillar ones.

Materials and Methods

Tissue preparation

All animal care and experimental procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the Animal Use Committee of Semmelweis University, Budapest and the Institute of Experimental Medicine, Hungarian Academy of Sciences. Hemicochlea preparations were carried out as described by the Dallos' lab [31–34]. Acutely dissected cochleae of CD-1 mice from postnatal day 15 (P15) to P21 were used. The onset of hearing in mice is around P10-14 [35]. Majority of physiological and structural bases of mice hearing over this age are considered mature [36–39].

Following decapitation, the head was divided in the medial plane and the bullae were removed. The bullae were placed in standard experimental solution (composition in mM: NaCl 150; KCl 3.5; $CaCl_2$ 1; $MgCl_2$ 1; Hepes 7.75; Tris 2.25; glucose 5.55; pH 7.4; 320 mOsm/l), that was continuously gassed with O_2. One of the bullae was opened under a stereomicroscope (Olympus SZ2-ST, Olympus Corporation, Philippines) and the cochlea was exposed. The cochlea was dissected from its surrounding bony structures with two forceps,

leaving the semicircular canals in place. The medial surface of the cochlea was dried with a small piece of filter paper, and glued (Loctite 404, Hartford, CT) onto a plastic plate with the diameter of 7 mm. Then the cochlea was placed into the cutting chamber of a vibratome (Vibratome Series 1000, Technical Products International Inc., St. Louis, Mo, USA) bathed again into the experimental solution, and cut into two halves through the middle of the modiolus with half of a double-edged razor blade (Wilkinson Sword GmbH, Germany). Only the half, glued to the plastic plate was used for imaging. By means of the plastic plate the preparation could be easily handled and mounted to the micromanipulator holder.

Calcium imaging

The whole procedure was performed at room temperature (22-24 ˚C). First, hemicochleae were incubated with the membrane-permeable AM ester derivative of fura-2 (10 µM) in the presence of pluronic F-127 (0.05%, w/v) for 30 min, then deesterified in standard experimental solution for 15 minutes before recording, i.e., rinsed three times in the loading chamber and perfused in the imaging chamber on the microscope stage. Proper positioning of the preparation in the imaging chamber under the microscope objective was ensured by a micromanipulator. The perfusion speed was 3.5 ml/min and the fura-2 loaded hemicochlea was alternately illuminated by 340 ± 5 nm and 380 ± 5 nm excitation light (Polychrome II monochromator, TILL Photonics, Germany) during imaging. The emitted light was monitored after passage through a 510-nm cut-off filter (20 nm band-pass). Fluorescent images were obtained with an Olympus BX50WI fluorescence microscope (Olympus, Japan) with a LUMPlanFl 40x/0.80w water immersion objective (Olympus, Japan), equipped with a Photometrics Quantix cooled CCD camera (Photometrics, USA). The system was controlled with the Imaging Workbench 4.0 software (INDEC BioSystems,

USA). The image frame rate was 1-2/sec during the ATP- and CPA-evoked responses and 0.03-0.1/sec otherwise to reduce UV illumination of the dye and the tissue.

The use of a 40x objective allowed the visualization of the organ of Corti in only one cochlear turn in the preparation. It is well known that many properties of cells in the organ of Corti is determined by their position along the cochlear spiral. We imaged supporting cells in the basal turn of the cochlea throughout this study.

The loading efficiency varied between cells, similarly to what is generally experienced with bulk loading of AM dyes, e.g., with fura-2 AM in brain slice preparations [40, 41]. Cells in 1-3 layers down the cut surface of the hemicochlea were used for fluorescence imaging because the signal detection of the fluorescent light was efficient from this depth. The focal plane of the experiments was chosen to include the utmost pillar, Deiters' and Hensen's cell with sufficient loading. Regions of interest surrounding the whole cells were used to measure average signal intensity and calculate $[Ca^{2+}]_i$ (see Data analysis).

Integrity of the preparations was assessed by the gross anatomy, the shape and location of the cells, the basal-, tectorial- and the Reissner's membranes and only the intact hemicochleae were used for functional imaging measurements [33, 42]. Dallos and his coworkers showed that various cellular structures in the preparation appeared to be viable within 1.5-2 h after dissection [33]. Our measurements were typically performed within 1.5-1.9 h. In some experiments, where four different ATP concentrations were tested in the same cells (Fig. 2A) the recordings lasted up to 2.2 h. In addition to the morphological criteria, functional properties as reversibility, repeatability, dose dependency and recovery of the ATP response (see the respective sections of the Results) also supported the viability of the P15-21 mouse hemicochlea preparation and its applicability for functional Ca^{2+} imaging in this time window.

Drug delivery

ATP was added to the perfusion for 30 sec, which caused a characteristic, reversible and repeatable response. The volume of buffer in the hemicochlea chamber was about 1.9 ml. The estimated ATP concentration at the site of the preparation in the chamber was about six times lower than in the perfusion buffer of ATP as estimated by dilution of phenol red. Cyclopiazonic acid (CPA) and Ca^{2+}-free (+ 1 mM EGTA) solution were started to apply to the perfusion after the end of the 1st ATP response and at least 15 min before the 2nd one.

Data analysis

The ratio of emitted fluorescence intensity (F_{340} / F_{380}) was calculated and converted into absolute values of $[Ca^{2+}]_i$. Cell image intensities were background-corrected using a nearby area devoid of loaded cells. Values of $[Ca^{2+}]_i$ in the cells were calculated off-line using the following equation: $[Ca^{2+}]_i = K_d \times F_{max380} / F_{min380} \times (R - R_{min}) / (R_{max} - R)$, where R is the actual ratio of emission intensity at 340 nm excitation to emission intensity at 380 nm excitation, R_{min} and R_{max} are the same ratios at 0 mM or saturating $[Ca^{2+}]$, respectively and F_{max380} and F_{min380} are the fluorescence intensities for 0 mM or saturating $[Ca^{2+}]$ at 380 nm excitation, respectively [43]. The parameters K_d, F_{max380} / F_{min380}, $R_{min,}$ and R_{max}, which characterize the system, were determined empirically by means of the Calcium Calibration Buffer Kit with Magnesium #2. Ca^{2+} transients were measured as the peak amplitude of ATP-evoked elevation of intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$ in nM; peak - basal; basal means average baseline $[Ca^{2+}]_i$ obtained during a 30-60 sec period prior to the respective ATP stimulation). Effect of drugs (and Ca^{2+} withdrawal) were expressed as the ratio of ATP response in the presence ($\Delta[Ca^{2+}]_{i,2}$) over the absence ($\Delta[Ca^{2+}]_{i,1}$) of the drug

($\Delta[Ca^{2+}]_{i,2}$ / $\Delta[Ca^{2+}]_{i,1}$). Desensitization was characterized similarly, i.e., the 2nd ATP transient was related to the 1st one. Normalizing the effect to the 1st response decreases the cellular variability (internal standard arrangement). Data are presented as mean ± standard error of the mean (SEM). Number of experiments (n) shows the number of individual cells. Every treatment group had cells from at least four mice. One-way ANOVA with Bonferroni post hoc test were used to determine the significance of data. In the experiments analysing the effect of both repetition time of ATP application and cell type on desensitisation two-way ANOVA followed by Bonferroni post hoc test was used. *$p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

RT-PCR detection

Twenty CD-1 mice (P15-19) were decapitated, and the bullae were removed from the skull. After opening the cochlea, the whole organ of Corti was removed from the bony modiolus under the stereomicroscope. The stria vascularis was peeled off, as well. The tissue was immediately collected into Eppendorf tubes cooled on dry ice, then stored at -80 ºC till analysis. In order to decrease the preparation time, only the organs of Corti of one side per mouse were collected. Total RNA from mouse cochlea samples was isolated with Trizol isolation reagent according to the protocol provided by the supplier (Invitrogen Life Technologies, Rockville, MD USA). RNA (2µl) was reverse transcribed with RevertAid First Stand cDNA Synthesis Kit (Invitrogen Life Technologies) as described in previous studies [44, 45]. Primers for amplification of P2X and P2Y receptor cDNAs were the following: for P2X1 (Fwd) 5'-CCT TGG CTA TGT GGT GCG AGA GTC, (Rev) 3'-AGG CAG GAT GTG GAG CAA TAA GAG; P2X2 5'-ATG GTG CAG CTG CTC ATT, 3'-AAA CGT GCA GTG CTT CAG; P2X3 5'-ATC AAG AAC AGC ATC CGT TTC CCT, 3'-AGT GTT GTC TCA

GTC ACC TCC TCA; P2X4 5'-ATC GTC ACC GTG AAC CAG ACA CA, 3'-CCA CGA TTG TGC CAA GAC GGA AT, P2X5 5'-TTT CTT CGT GGT CAC CAA CCT GAT, 3'-ATT TGT GGA GCT GAA GTG ACA GGT; P2X6 5'-CTG TGG GAT GTG GCT GAC TT, 3'-TCA AAG TCC CCT CCA GTC AT, P2X7 5'-CCA CAA CTA CAC CAC GAG AAA C, 3'-ACT TCT TGG CCC TTG ACA TCT T, P2Y1 5'-AAG ACC GGT TTC CAG TTC TAC TAC, 3'-CAC ATT TCT GGG GTC TGG AAA TCC; P2Y2 5'-TGC TGG TGC TGG CCT GCC AGG CAC, 3'-GCC CTG CCA GGA AGT AGA GTA CCG; P2Y4 5'-ATG AGG ATT TCA AGT TCA TCC TGC, 3'-TAG ACC ACG TTG ACA ATG TTC AGT; P2Y6 5'-CTG CGT CTA CCG TGA GGA TT, 3'-GCT ATG AAG GGC AGC AAG AA; P2Y12 5'-CAG GTT CTC TTC CCA TTG CT, 5'-CAG CAA TGA TGA TGA AAA CC; P2Y13 5'-ATC TTG AAC AAG GAG GCA A, 5'-TCT TTT TAC GAA CCC TGT T; P2Y14 5'-TAG AGG CCA TAA ACT GTG CTT, 5'-AAT TCT TCC TGG ACT TGA GGT; β-actin 5′-AGC TGA GAG GGAAATCGTGC-3′, 5′-GAT GGA GGG GCC GGA CTC AT-3′.

The conditions for amplification were as follows: initial denaturation at 95 ˚C for 5 min, hot start at 80 ˚C, then 94 ˚C for 1 min, 59 ˚C for 1 min, and 72˚C for 1 min for 40 cycles, with a final extension at 72 ˚C for 5 min. PCR products were analyzed by agarose gel electrophoresis.

Materials

Fura-2 AM, Pluronic F-127 and Calcium Calibration Buffer Kit with Magnesium #2 was obtained from Molecular Probes, USA, cyclopiazonic acid from Alomone Labs, Israel. All other chemicals were purchased from Sigma-Aldrich, USA.

Results

Ca^{2+} imaging of pillar, Deiters', and Hensen's cells in hearing mouse hemicochlea

To investigate the mechanism of Ca^{2+} signaling and the regulatory role of ATP in different supporting cell types of the organ of Corti we developed a reliable method for labeling individual cells and measuring $[Ca^{2+}]_i$ in the unique hemicochlea preparation of P15-21 mice [31–34]. The hemicochleae were bulk loaded with fura-2 AM, a ratiometric, high-affinity Ca^{2+} indicator (Fig. 1A). Only preparations with intact morphology were used (see Materials and Methods). Cells were identified based on their anatomical location and shape under a 40x objective with red light oblique illumination.

We determined the basal, resting $[Ca^{2+}]_i$ of the supporting cells in hearing mice at the beginning of every recordings. Fig. 1B shows that the average resting $[Ca^{2+}]_i$ in the pillar (61 ± 4 nM, n = 41) and Deiters' cells (58 ± 5 nM, n = 65) was significantly lower than in the Hensen's cells (98 ± 10 nM, n = 53). Notably, we found nearly 2 folds higher resting $[Ca^{2+}]_i$ in the Hensen's cells, compared with the other two cell types suggesting a cell-type specific intracellular Ca^{2+} handling mechanism.

ATP evoked reversible and repeatable Ca^{2+} signals in the supporting cells in a dose-dependent manner

In order to test whether ATP, an important regulator of Ca^{2+} signaling in the cochlea, is able to evoke changes in the $[Ca^{2+}]_i$ in supporting cells of the organ of Corti in the mature hemicochlea, we applied ATP for 30 sec in bath perfusion.

ATP evoked characteristic, intracellular Ca^{2+} transients in a dose-dependent manner in the tested 1 – 100 µM range in all three types of supporting cells. The ATP responses were reversible and repeatable (Fig. 2A, upper traces). Pillar cells showed the lowest sensitivity for ATP, as 1 and 10 µM of the nucleotide evoked the smallest transients in this supporting cell type (vs. the Deiters' and Hensen's cells; Fig. 2A, bar graphs). Application of 50 µM ATP induced a fast rising, uniformly shaped Ca^{2+} transient reliably in all three types of supporting cells ($\Delta[Ca^{2+}]_i$ in nM; pillar cells: 96 ± 14 nM, n=41; Deiters' cells: 104 ± 9 nM, n=65; Hensen's cells: 140 ± 10 nM, n=53), therefore we used this concentration of ATP in further experiments. We did not observe any contraction based movement in the preparation after ATP application (not even in Deiters' cells).

Upon repeated application, the ATP response showed a reduction, in inverse correlation with the time interval between ATP administrations. There was no difference between the cell types in this respect (Fig. 2B, bar graphs). The reduction was negligible when the ATP applications followed each other by 20 min (pillar cells: 3 ± 16 %, n = 9; Deiters' cells: 6 ± 4 %, n = 14; Hensen's cells: 13 ± 4 %, n = 20). Compared to that, a significant reduction in the transients were seen with 5 min intervals in Deiters' and Hensen's cells (34 ± 8 %, n = 20 and 51 ± 12 %, n = 8, respectively). The tendency of reduction in the transients was also evident in pillar cells (40 ± 9 %, n = 11), although the difference between the 20-min and the 5-min-responses was not statistically significant (Fig. 2B, bar graphs). The 3rd applications of ATP has confirmed (Fig. 2B, representative traces), that while 5 min repetition of ATP resulted in pronounced desensitization of the ATP-evoked Ca^{2+} response, 20 min was enough for the transient to be recovered. In further experiments, we repeated the ATP stimuli with 20 min intervals.

ATP-evoked Ca^{2+} transients were mediated by Ca^{2+} influx and release of Ca^{2+} from internal stores in a cell-type specific manner

The calcium ions, building up the ATP-evoked transients, may originate from both extra- and intracellular sources. To explore their involvement we tested the effect of ATP in Ca^{2+}-free buffer and after depletion of the SERCA-dependent intracellular Ca^{2+} stores in an internal standard type of experimental design (2nd ATP stimulus presented during perturbation of extra- or intracellular Ca^{2+} sources; see Fig. 3A and B and in Materials and Methods).

Ca^{2+}-free medium (+ 1 mM EGTA) suppressed the ATP-evoked intracellular Ca^{2+} signals significantly in all three types of cells (Fig. 3A and C), i.e., the $\Delta[Ca^{2+}]_{i,2}$ / $\Delta[Ca^{2+}]_{i,1}$ ratios were decreased. The inhibition was more pronounced in the Deiters' and the Hensen's cells (22 ± 8 and 22 ± 4 % of the 1st ATP response, respectively) compared to the pillar cells (38 ± 14 % of the 1st ATP response; Fig. 3C). Recovery of the Ca^{2+} transients for the 3rd ATP stimulus after readministration of the normal solution (data not shown) indicated that the cells preserved their integrity and responsiveness.

The Ca^{2+}-free medium did not change the resting $[Ca^{2+}]_i$ in either cell type, i.e., the comparison of the resting $[Ca^{2+}]_i$-s, measured in the presence and absence of extracellular Ca^{2+} right before the 1st and 2nd ATP stimulus, respectively, did not show any significant difference (data not shown).

The intracellular Ca^{2+} stores were depleted by the specific SERCA inhibitor CPA (10 µM), which inhibits store refilling (Fig. 3B and C). Empty stores hampered the ATP-evoked transients significantly in all three cell types, i.e., the $\Delta[Ca^{2+}]_{i,2}$ / $\Delta[Ca^{2+}]_{i,1}$ ratios were decreased. Again, the effect was more robust in the Deiters' and Hensen's cells (18 ± 4 and 8 ± 3 % of the 1st ATP response, respectively) than in the pillar ones (33 ± 8 % of the 1st ATP response; Fig. 3C). Recovery of the Ca^{2+} transients for the 3rd ATP stimulus after

readministration of the normal (no CPA) solution (data not shown) indicated that the cells preserved their integrity and responsiveness.

CPA may also be used for characterization of SERCA-dependent intracellular Ca^{2+} stores by revealing their leakage in the absence of refilling. Indeed, CPA itself, before the 2nd ATP application, increased the $[Ca^{2+}]_i$ in all three cell types (Fig. 3B). There was a modest effect in pillar cells and more pronounced in Deiters' and Hensen's cells, suggesting a difference in the regulation of $[Ca^{2+}]_i$ in these supporting cell types, as well (Fig. 3D).

mRNAs of both P2X and P2Y receptor subtypes were expressed in the organ of Corti

In order to determine the possible subtypes of P2 receptors that may be involved in the action of ATP, we measured the mRNA expression of P2X and P2Y receptor subunits in the excised organ of Corti of P15-19 CD-1 mice. The RT-PCR analysis showed the presence of the mRNA of P2X2, P2X3, P2X4, P2X6, P2X7 and P2Y1, P2Y2, P2Y6, P2Y12, P2Y14 receptors in the whole organ of Corti (Fig. 4).

Discussion

Intracellular Ca^{2+} signals universally serve as second messengers [46], regulating a variety of intra- and intercellular processes also in the organ of Corti [8]. The intracellular Ca^{2+} signaling of the cochlear cells are controlled or affected by ATP through purinergic receptors during maturation, physiological sound transduction and under pathological conditions, as well [4, 5, 47–49].

Besides a hormone-like tonic regulation, based on sound exposure-induced release of ATP from the stria vascularis [50] locally released ATP, as an auto- and paracrine regulator, can modulate purinergic activity in the organ of Corti. ATP, escaped from injured hair cells induces intercellular Ca^{2+} signaling among the supporting cells [4, 18]. Furthermore, the supporting cells themselves are able to release ATP into the extracellular space through connexin hemichannels [13]. This kind of ATP-mediated paracrine signaling was previously observed in glia and glia-like tissue as well [51–53].

Investigation of the ATP-regulated Ca^{2+} signaling is predominantly performed in isolated cells [22–26] or cochlear explants from embrionic or newborn murines [4, 18, 27], experimental models which lacks normal tissue organization or contaminated by developmental biological factors. The advantage of our approach is that it allows the comparison of calibrated $[Ca^{2+}]_i$ values of three different supporting cell types (pillar, Deiters' and Hensen's) investigated in the same *in situ* preparation from mature hearing mice [35].

The real physiological concentration of ATP directly at the release site and nearby the receptors is only predicted, because of unresolved methodological challenges [54]. That is also the case in the organ of Corti [38]. In tissue-cultured supporting cells of newborn animals, locally applied ATP in a nanomolar range elicited repeatable intra- and intercellular Ca^{2+} oscillation that turned to a slowly declining Ca^{2+} response above few µM concentrations of ATP [4, 18]. A broad concentration range of ATP was tested (0.01 – 1000 µM) and used (predominantly 100 µM and 10 µM) in different studies in dissociated supporting cells isolated from mature cochleae [23–26]. The ATP induced intracellular Ca^{2+} transients were also shown in Deiters' [30] and Hensen's [29] cells in *in situ* preparation from adult guine-pigs, where EC_{50} for ATP was ~ 50 µM in Hensen's cells and properties of the 100 µM (puff from pipette) and 1 mM (caged) ATP evoked transients were investigated further. Direct comparison of ATP sensitivity of the supporting cells in different studies is encumbered by

the different preparations used and the different ways of ATP applications (different puffs, perfusions and caged release). In our *in situ* hemicochlea preparation from hearing mice, ATP evoked reversible and repeatable Ca^{2+} transients in a dose dependent manner in the 1 – 100 µM range in all the investigated supporting cell types (pillar, Deiters' and Hensen's cells). Considering the method of our ATP application (in short perfusion, see Materials and Methods) there is some overestimation of ATP concentration that really reached the receptors on the cells, i.e., the sensitivity of the cells for ATP is supposed to be somewhat higher. However, our data, as they were collected from the three different supporting cells under identical conditions, may show reliably the bit lower sensitivity of pillar cells, which produced an insignificant Ca^{2+} response for 10 µM ATP, contrary to Deiters' and Hensen's cells.

The amplitudes of the evoked transients and basal Ca^{2+} concentrations we measured in absolute values (nM) are in the same magnitude published for dissociated Deiters' and Hensen's cells of adult guinea-pigs [23, 25], but thorough comparisons are halted by the fact that other studies rather used uncalibrated ratio or $\Delta F/F$ values of single wavelength dyes.

Repeating the stimulus in 5 and 10 mins showed desensitization of the ATP response in all three cell types what have already been observed in isolated Deiters' and Hensen's cells [23, 25]. We have not investigated the mechanism underlying desensitization in this study. Decrease in the amplitude of subsequent responses disappeared at 20 min stimulation interval, providing the opportunity for internal standard experimental arrangement (see Materials and Methods).

A straightforward way of separating the ionotropic P2X and the metabotropic P2Y receptor-mediated components of ATP-evoked Ca^{2+} responses from each other is withdrawing Ca^{2+} from the extracellular buffer and depleting intracellular Ca^{2+} stores by blocking their SERCA pump, respectively. In our experiments both intervention, omission of

Ca^{2+} and application of CPA, inhibited the response, suggesting the involvement of both the ionotropic- and the metabotropic ATP receptors, in all three cell types. This conclusion, although without cell specificity and not on the protein level, was supported by the presence of the mRNA of $P2X_{2,3,4,6,7}$ and $P2Y_{1,2,6,12,14}$ receptors in the organ of Corti of the same preparation.

The inhibitory effect of Ca^{2+} withdrawal on the ATP-evoked Ca^{2+} transients in pillar, Deiters' and Hensen's cells was shown in different experimental models of largely isolated cells [23–26] and/or in situ preparation [30, 29], but neither of these studies investigated all three types of supporting cells in the same preparation synchronously. Probably this is the reason of the relatively broad published range of efficiency of Ca^{2+} omission in inhibiting the effect of ATP, including even the total abolishment of the response in Hensen's cells [23]. The effect of depletion of endoplasmic reticulum (ER) Ca^{2+} stores was investigated much sparsely and in P1-3 rat organ culture preparation [4, 18]. The measurement of the effect of both interventions in the same study, especially in all three cell types in the same preparation, was not performed hitherto, according to our best knowledge.

P2X receptors are ligand-gated ion channels permeable for cations like Ca^{2+} and K^+ [7, 55]. It seems that P2X receptors are responsible for K^+ shunting from the endolymph to the perilymph through the cells [15] adjusting the hearing sensitivity by declining the endocochlear potential to reduce the possibility of cell damage. Activation of these receptors induces uncoupling among the supporting cells, thought to suppress K^+ flow back into the endolymphatic K^+ cycle via the neighboring cells [56]. P2X2 is supposed to be the dominant ionotropic purinergic receptor in the supporting cells, while the expression of some other types of P2X receptors showed age-dependency with transient appearance [58–63]. However, a recent report based on human genetical and functional observations confirmed a functional role of P2X2 receptors in human adults, demonstrating that non-functioning mutation of P2X2

receptors in the cochlea leads to progressive hearing loss with the onset of 12 to 20 years, but not to congenital deafness or severe hearing problems [64]. This early presbycusis was exacerbated by noise suggesting that P2X2 receptors are required for life-long normal hearing and protection against noise exposure. This is in accordance with the upregulation of P2X2 receptor expression observed in noise exposure in the organ of Corti [65], which capacity decreases with age in adult animals [66].

We found that, besides the contribution of extracellular Ca^{2+}, the ATP-evoked Ca^{2+} transients were also dependent on the intracellular Ca^{2+} stores, but more strongly in the Deiters' and Hensen's cells than in the pillar ones. It has been shown previously that SERCA pump inhibition by thapsigargin or CPA unmasks the leak of Ca^{2+} from the ER in different cell types [67, 68] including glial cells [69]. The magnitude of the leak characterizes the capacity and permeability of the stores and influences their filling state which affects the formation of cytosolic Ca^{2+} signals [70]. Thus the more pronounced leak from the ER in Deiters', and especially in the Hensen's cells may also indicate the higher activity of the internal stores in Ca^{2+} signaling in these cells compared to the pillar ones. The highest leak in Hensen’s cells may be related to the higher basal $[Ca^{2+}]_i$ of this cell type that could promote the loading of intracellular stores.

While in pillar cells the sum of the inhibited portion of the ATP response in the absence of extracellular Ca^{2+} and in the presence of CPA approximated the amplitude of the control response, the sum of the respective inhibitions in Deiters' and Hensen's cells definitely surpassed that. This supralinear additivity of the extracellular Ca^{2+}- and Ca^{2+} store-dependent ATP responses, versus the linear additivity in pillar cells, suggests a synergistic interaction between the extracellular Ca^{2+}- and intracellular store-dependent ATP signaling in Deiters' and Hensen's cells. The interaction may reflect calcium-induced calcium release (CICR), where depletion of stores would not simply inhibit the metabotropic P2Y receptor-mediated

response, but also prevents the amplification by the Ca^{2+} stores upon P2X receptor activation. And vice versa, omission of Ca^{2+} from the extracellular space would not simply inhibit the ionotropic P2X receptor-mediated response, but also abolish the Ca^{2+} influx that triggers the release of Ca^{2+} from intracellular stores. This might also explain the massive inhibitory effect of CPA, which seemed even more pronounced than that of the Ca^{2+} withdrawal.

In CICR Ca^{2+} activates either ryanodine receptors or IP_3 receptors that are Ca^{2+} channels of the internal stores. Traditionally, CICR is considered to phenomenon based on ryanodine receptors (RyRs). Ca^{2+} release from the ER through IP_3 receptors is not depending exclusively on Ca^{2+} alone, but also on the presence of IP_3 [71]. CICR based on RyR has already been observed in the cochlea in hair cells and spiral ganglion neurons [72–74], but the role of RyR is controversial in the glia-like cochlear supporting cells. Piazza et al. showed that RyRs are not involved in the purinergic signaling among the supporting cells of rat pups [18], while it was demonstrated that RyR2 proteins are strongly expressed in the cup region of Deiters' cells in adult rats [76], the intercellular coupling in the Hensen's cells can be influenced by the RyR agonist caffeine and ryanodine [77], and the cochlear micromechanics is affected by these two drugs in young guinea pigs [78]. A more recent report by Liang et al. [79] may explain all these findings by showing the age-dependency of RyR expression. They have found that RyR receptors were missing in the supporting cells of newborn rats, but at the age of P10, weak expression of RyRs was present in all types of supporting cells at the lesser epithelial ridge, and an even more strong expression was observed in adult animals. Thus, the involvement of RyR-dependent CICR in the ATP-evoked Ca^{2+} signaling in the Deiters' and Hensen's cells is a reasonable possibility.

IP_3-dependent CICR can be present in those cells in which intracellular Ca^{2+} signaling is largely IP_3-dependent [80]. In these cell types the initial Ca^{2+} release sensitizes the neighboring ER pools to IP_3, resulting in a CICR-like signal propagation [81]. IP_3 was shown

to be an important intercellular signaling molecule in the organ of Corti. Disturbances in its production impairs hearing [82], and a mutation that reduced the permeability of IP_3 through gap junctions was proven to be resulted in deafness [19]. The IP_3 receptor-dependent CICR is also in accordance with our findings of crucial involvement of internal Ca^{2+} stores and a CICR-like phenomenon in ATP-evoked Ca^{2+} signaling in the Deiters' and Hensen's cells.

Our results suggest that the ATP-evoked Ca^{2+} signaling is quite similar between Deiters' and Hensen's cells, contrary, for example to Dulon et al., 1993, who showed a differential Ca^{2+} response to ATP between Deiters' and Hensen's cells, i.e., no release of Ca^{2+} from internal stores in the Hensen's cells. The pillar cells, which are not innervated [24] like Deiters' and Hensen's cells [83, 84] resembles less to the other two supporting cells of the organ of Corti in this respect.

Conclusion:

Here we have demonstrated that Ca^{2+} imaging in the *in situ* hemicochlea preparation of hearing mice is a reliable method to characterize ATP-evoked Ca^{2+} signaling in different supporting cell types of the organ of Corti. Our results reflect more closely the adult in vivo situation than the ones acquired in isolated cells or explants from rodents with immature hearing and provides experimental condition for reliable comparison of different supporting cell types. We measured the basal $[Ca^{2+}]_i$ and the leak of Ca^{2+} from SERCA-dependent internal stores and demonstrated the ATP signaling in pillar, Deiters' and Hensen's cells of the organ of Corti and suggested the involvement of both the ionotropic P2X and the metabotropic P2Y receptors and, in the case of Deiters' and Hensen's cells, the possible CICR-based synergistic interaction of the two signaling pathways. Differences in the Ca^{2+} signaling of the different supporting cell types may reflect their distinct role in cochlear pathophysiology.

Acknowledgments

This work was supported by the Hungarian-French Collaborative R&I Programme on Biotechnologies (TÉT_10-1-2011-0421). We thank Peter Dallos and Claus-Peter Richter for teaching us the preparation of the hemicochlea.

Figure legends

Fig. 1. Calcium imaging of the supporting cells in the hemicochlea preparation of hearing mice

A) The upper image shows the organ of Corti in the basal turn of the cochlea by oblique illumination. The lower fluorescent images were taken in the same preparation at 340 and 380 nm excitation after bulk loading by fura-2 AM. TM, tectorial membrane; BM, basilar membrane; IHC, inner hair cell; PC, pillar cell; OHC, outer hair cell; DC, Deiters' cell; HC, Hensen's cell. Scale bars represent 20 µm.

B) Basal $[Ca^{2+}]_i$ in different supporting cell types of the organ of Corti. Note the higher resting intracellular Ca^{2+} concentration in the Hensen's cells (n = 53) compared to the pillar (n = 41; $^{**}p < 0.01$) and Deiters' cells (n = 65; $^{***}p < 0.001$).

Fig. 2. ATP evoked reversible and repeatable intracellular Ca^{2+} transients in pillar, Deiters' and Hensen's cells

A) Upper traces: ATP evoked intracellular Ca^{2+} transients in each type of supporting cells in a dose-dependent and repeatable manner. Representative traces show the responses for increasing doses of ATP (1, 10, 50 and 100 µM; 30 sec perfusion; black dots), applied with 20 min intervals in the same cell. The scale bars indicate the change of $[Ca^{2+}]_i$ and the time. Lower bar graphs: Mean + SEM of the Ca^{2+} transients evoked by different concentrations of ATP in the three supporting cell types. 1 µM (light gray bars), 10 µM (gray bars), 50 µM (dark gray bars) and 100 µM (black bars) of ATP. Pillar cells, n = 4, 3, 41, 2; Deiters' cells, n = 9, 9, 65, 8; Hensen's cells, n = 2, 2, 53, 3.

B) Upper traces: Representative traces of intracellular Ca^{2+} transients evoked by consecutive perfusion (30 sec) of 50 µM ATP (black dots). The ATP responses were reversible and

repeatable in all three cell types, but repeating the application of ATP in 5 min resulted in the reduction of the transients, while leaving 20 min before the next application allowed the response to recover. The scale bars indicate the change of $[Ca^{2+}]_i$ and the time.

Lower bar graphs: Reduction of the 2nd ATP-evoked (50 µM) Ca^{2+} transients was dependent on the time intervals between repetitions in all three cell types (20, 10 and 5 min; black, dark and light gray bars, respectively). The respective 5 min-values differed significantly from the 20-min-ones in Deiters' and Hensen's cells and pillar cells also showed a clear tendency of desensitization. Bars represent the mean ± SEM of the ratio of the 2nd to the 1st ATP-evoked Ca^{2+} transients ($\Delta[Ca^{2+}]_{i,2}$ / $\Delta[Ca^{2+}]_{i,1}$). Pillar cells, n = 9, 6, 11; Deiters' cells, n = 14, 9, 20 and Hensen's cells, n = 20, 4, 8. *$p < 0.05$; **$p < 0.01$.

Fig. 3. ATP-evoked intracellular Ca^{2+} transients are extracellular Ca^{2+} and intracellular Ca^{2+} store dependent in the supporting cells of the organ of Corti

A-B) The representative traces show the effect of the omission of extracellular Ca^{2+} (+ 1 mM EGTA) and perfusion of 10 µM CPA on the ATP-evoked Ca^{2+} transients in the different supporting cells. Black dots indicate the application of ATP (50 µM). Ca^{2+}-free and CPA were administered as indicated by the horizontal lines.

C) Effect of the withdrawal of Ca^{2+} from the buffer (+ 1 mM EGTA; Ca^{2+}-free) and 10 µM CPA on the transients evoked by 50 µM ATP. The interval between the ATP application was 20 min. Bars represent the mean ± SEM of the ratio of the Ca^{2+} transients in the presence (2nd ATP response) and in the absence (1st ATP response) of Ca^{2+} omission/CPA ($\Delta[Ca^{2+}]_{i,2}$ / $\Delta[Ca^{2+}]_{i,1}$). Pillar cells, n = 9, 7, 8; Deiters' cells, n = 14, 12, 10 and Hensen's cells, n = 20, 14, 7. *$p < 0.05$; **$p < 0.01$, ***p < 0.001.

D) CPA (10 μM) increased the basal $[Ca^{2+}]_i$ in all three supporting cell types, slightly in pillar (n = 8) and more in Deiters' (n = 10) and Hensen's cells (n = 7; *$p < 0.05$ compared to pillar cells). Bars represent the mean + SEM of the peak responses in nM.

Fig. 4. RT-PCR analysis reveals the expression of multiple P2X and P2Y receptor subtypes in the organ of Corti of hearing mice

Total RNA samples from organs of Corti of one side of twenty P15-19 CD-1 mice each were reverse transcribed and amplified by PCR using primers specific to P2X and P2Y receptor transcripts. Amplification of β-actin was used as an internal control. The identity of the amplified PCR products has previously been verified by sequencing (Sperlágh et al., 2003). A 100-bp DNA ladder (Fermantas, Vilnius, Lithuania) was used to identify PCR fragment sizes (st). The gel shown is representative of three independent analysis. mRNAs encoding P2X2, P2X3, P2X4, P2X6, P2X7, and P2Y1, P2Y2, P2Y6, P2Y12, P2Y14 receptors (black letters) were present in the organ of Corti.

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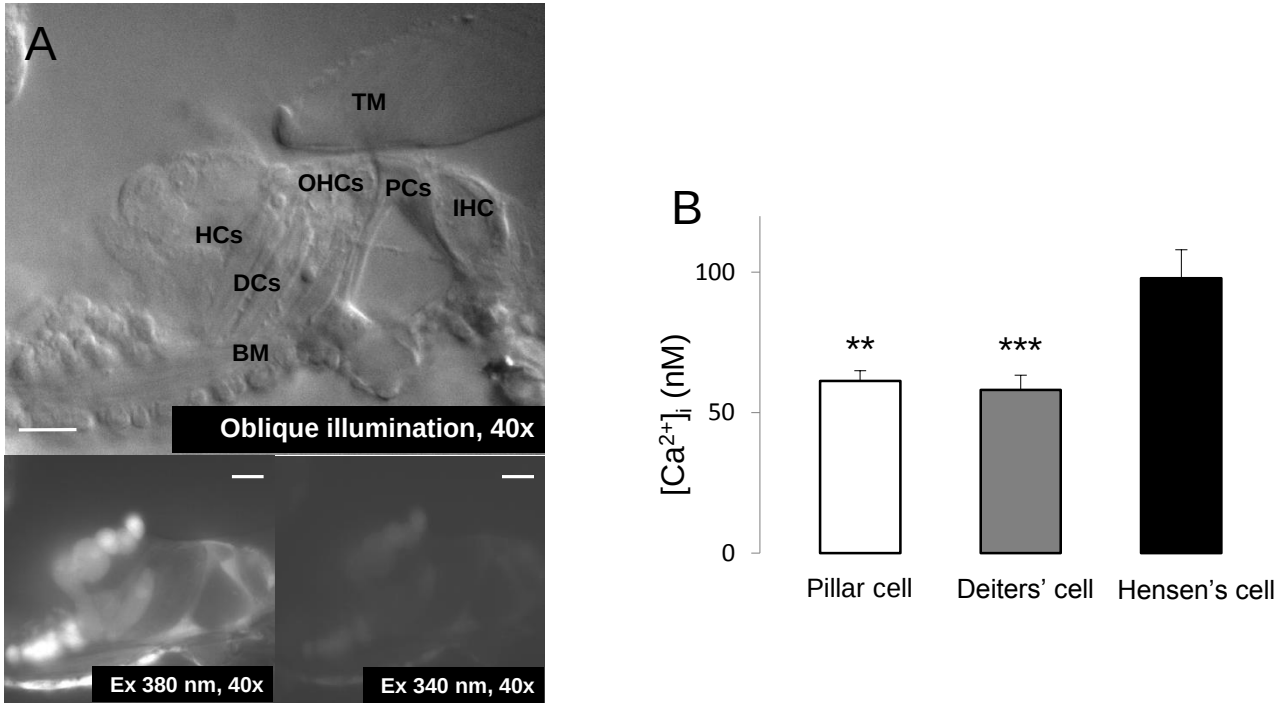

Figure 2

Fig. 2

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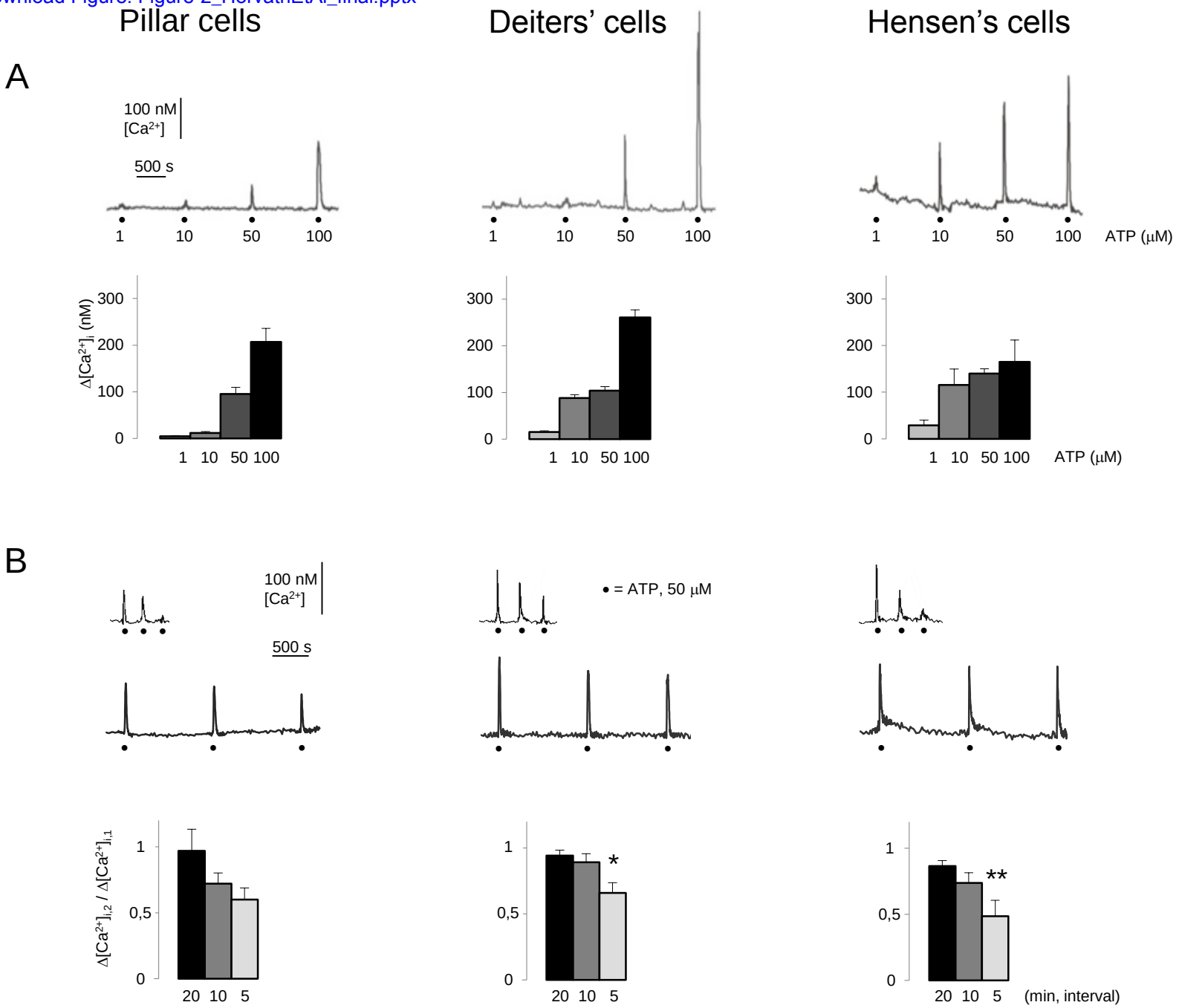

Figure 3
Fig. 3
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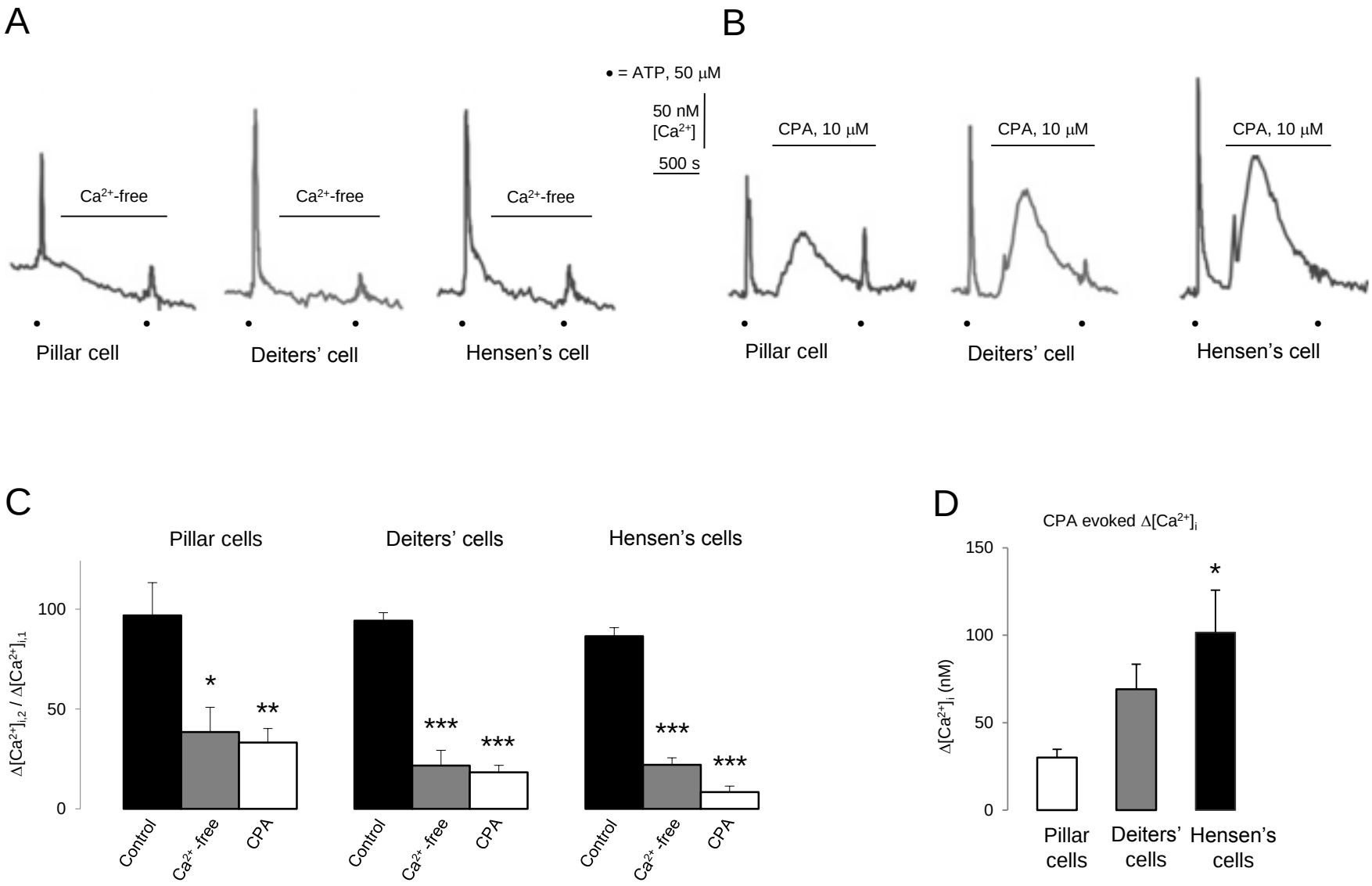

Figure 4

Fig. 4

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