Effects of SERCA and PMCA inhibitors on the survival of rat cochlear hair cells during ischemia *in vitro*

Running title: Ischemia-induced hair cell loss

Nyamaa Amarjargal[1], Birgit Mazurek[1], Heidemarie Haupt[1], Nadeshda Andreeva[2], Julia Fuchs[1], Johann Gross[1]

[1]Molecular Biological Research Laboratory, Department of Otorhinolaryngology, Charité - University Medicine Berlin, Campus Charité Mitte, Berlin, Germany
[2]Brain Research Institute, Academy of Medical Sciences, Moscow, Russia

Corresponding author: Prof. Dr. Johann Gross, Molecular Biological Research Laboratory, Department of Otorhinolaryngology, Charité - University Medicine Berlin, Campus Charité Mitte, Charitéplatz 1, 10117 Berlin, Germany, Tel.: +49-30-450555311; Fax +49-30-450555 908, E-mail address: johann.gross@charite.de

Summary

An important mechanism underlying cochlear hair cell (HC) susceptibility to hypoxia/ischemia is the influx of Ca^{2+}. Two main ATP-dependent mechanisms contribute to maintaining low Ca^{2+} levels: uptake of Ca^{2+} into intracellular stores via smooth endoplasmic reticulum calcium ATPase (SERCA) and extrusion of Ca^{2+} via plasma membrane calcium ATPase (PMCA). The effects of the SERCA inhibitors thapsigargin (10 nM-10 μM) and cyclopiazonic acid (CPA; 10-50 μM) and of the PMCA blockers eosin (1.5-10 μM) and o-vanadate (1-5 mM) on inner and outer hair cells (IHCs/OHCs) were examined in normoxia and ischemia using an *in vitro* model of the newborn rat cochlea. Exposure of the cultures to ischemia resulted in a significant mean loss of HCs. Thapsigargin and CPA had no effect. Eosin decreased the numbers of IHCs and OHCs by up to 25% in normoxia and significantly aggravated the ischemia-induced damage to IHCs at 5 and 10 μM and to OHCs at 10 μM. o-Vanadate had no effect on IHC and OHC counts in normoxia, but aggravated the ischemia-induced HC loss in a dose-dependent manner. The effects of eosin and o-vanadate indicate that PMCA has an important role to play in protecting the HCs from ischemic cell death.

Key words

Calcium • Organ of Corti • Ischemia • PMCA • Rat

Introduction

Hypoxia/ischemia is an important pathogenetic factor contributing to inner ear diseases. Sudden hearing loss, noise induced hearing loss and presbyacusis are believed to be associated with hypoxia/ischemia (Riva *et al.* 2007).

In the auditory system, Ca^{2+} participates in regulating several activities of the cochlear sensory hair cells (HCs), including depolarization and repolarization, neurotransmitter release, adaptation and HC motility (Crawford *et al.* 1991, Lewis and Hudspeth 1983). These functions are provided by different Ca^{2+} concentrations in the fluids of the internal ear, the perilymph (PL) and the endolymph (Bosher and Warren 1978), which are presumably maintained by active processes (Furuta *et al.* 1998). Ischemia-induced neuronal cell death was shown to be largely determined by increases in the intracellular Ca^{2+} concentration (Wang *et al.* 2002). The increase of intracellular Ca^{2+} levels has several consequences: activation of Ca^{2+}-regulated enzymes, mitochondrial Ca^{2+} overload, cytoskeletal disruption or activation of calpains (Lipton 1999, Missiaen *et al.* 2000). Excessive Ca^{2+} increase may lead to cell death via apoptosis or necrosis (Orrenius *et al.* 2003).

There are several processes which are involved in maintaining a low Ca^{2+} level within the cochlear HCs: regulated Ca^{2+} uptake, intracellular Ca^{2+} buffers (Slepecky and Ulfendahl 1993), intracellular compartmentalization (Tucker and Fettiplace 1995) and active extrusion (Ikeda *et al.* 1992). Ca^{2+} is continuously removed from the cells' cytoplasm via two ATP-dependent pathways: plasma membrane Ca^{2+} ATPase (PMCA) and smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA). The PMCA regulates the cytosolic Ca^{2+} concentration by extruding Ca^{2+} in a calmodulin-dependent manner (Carafoli 1997). Many studies have shown that PMCA is present in the HC bundles of the mammalian cochlea (Apicella *et al.* 1997, Crouch and Schulte 1995) where it is crucial for

regulating the Ca^{2+} ion level during transduction (Dumont *et al.* 2001, Yamoah *et al.* 1998). Recently, immunohistochemical studies have demonstrated that PMCA is expressed not only in the HC bundles of both inner and outer hair cells (IHCs/OHCs), but also in their basolateral membranes (Dumont *et al.* 2001).

The SERCA-type intracellular Ca^{2+} pump transports Ca^{2+} ions from the cytoplasm to the intracellular Ca^{2+} stores. In the IHCs, the intracellular Ca^{2+} ions ($[Ca^{2+}]_i$) can be taken up into intracellular stores and be released to modulate signal transduction. The SERCA seems to play a crucial role in compartmentalizing $[Ca^{2+}]_i$ signals (Kennedy 2002). In the HCs, the SERCA exerts modulating effects rather than displaying clearing activities (Evans *et al.* 2000, Kennedy 2002). In the OHCs, Ca^{2+} from intracellular stores contributes to increasing the acetylcholine (ACh)-evoked Ca^{2+} in the postsynaptic HC region by providing Ca^{2+} release in response to Ca^{2+} influx (Evans *et al.* 2000). Coupling between $[Ca^{2+}]_i$ stores and the Ca^{2+} permeability of the plasma membrane was reported (Mason *et al.* 1991). The action of ACh on the OHC current is fast and requires both extracellular and intracellular Ca^{2+} (Frolenkov *et al.* 2003). Ca^{2+} can also be extruded via Na^{+}-Ca^{2+} exchange using the energy from the Na^{+} gradient. This mechanism was found to be active in OHCs but with a low capacity (Ikeda *et al.* 1992). It was found to be inactive in IHCs (Kennedy 2002). The relative contribution of the Ca^{2+} clearance systems is not known for cochlear HCs.

To assess the contribution of Ca^{2+} uptake by SERCA and Ca^{2+} extrusion by PMCA to cochlear HC survival during ischemia, we examined the effect of the SERCA inhibitors thapsigargin and cyclopiazonic acid (CPA) and of the PMCA blockers eosin and o-vanadate at different concentrations on IHC and OHC loss in normoxia and ischemia using an *in vitro* model of the newborn rat cochlea (Gatto *et al.* 1995, Mazurek *et al.* 2003, Thastrup *et al.* 1990).

Methods

For this study, an *in vitro* model of the organ of Corti from 3-5 day old Wistar rats (n = 109) was used (Cheng *et al.* 1999, Lowenheim *et al.* 1999). The pups were surface-sterilized with 70% ethanol and decapitated. The left and right temporal bones were dissected in buffered saline glucose (BSG) plus ciprobay under sterile conditions. The otic capsule was removed and the membranous cochleae were prepared. Then, the modiolus and stria vascularis were removed from the organ of Corti and the specimens were divided into their apical, middle and basal parts.

The fragments were cultured in four-well microtiter plates (500 µl/well) in Dulbecco's modified Eagle's medium/F12 nutrient mixtures (DMEM/F12, Gibco, Karlsruhe, Germany) (1:1) medium with 10% fetal calf serum (FCS), 10 mM HEPES, 5 mM L-glutamine, 50 U/ml ciprobay, 100 µg/ml transferrin, 60 µg/ml putrescine, 25 µg/ml insulin, 0.6% glucose. The cultures were placed in an incubator at 37°C and were grown for overnight. For experimental incubation, an artificial PL like electrolyte solution (in mM: 125 NaCl, 5 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 1.99 EGTA, 20 Hepes, 24 $NaHCO_3$ and 10 glucose) (Bobbin *et al.* 2003, Wanaverbecq *et al.* 2003) was used.

Ischemia was mimicked by incubating the fragments in artificial PL without glucose (500 µl/well) in a Billups-Rothenberg chamber for 4 h. The chamber containing the plates was perfused with a calibrated gas mixture of 5% CO_2 and 95% N_2 (AGA Gas GmbH, Bottrop, Germany). After 15 min perfusion at a flow rate of 20 l/min, the pO_2 in artificial PL was 10-20 mm Hg, and remained at the same level during incubation. Controls were incubated in the same incubator for 4 h in artificial PL.

To study the effect of SERCA and PMCA inhibitors on the HCs, the cultures were grouped as follows: (1) controls, i.e., incubation in artificial PL in normoxia (n = 19); (2) ischemia, i.e., exposure to hypoxia in artificial PL without glucose (n = 23); (3) incubation

in artificial PL with increasing concentrations of thapsigargin (10, 100 nM and 1, 10 μM) in normoxia (n = 21) and ischemia (n = 22); (4) incubation in artificial PL with 10 and 50 μM CPA in normoxia (n = 14) and ischemia (n = 18); (5) incubation in artificial PL with increasing concentrations of eosin (1.5, 5, 10 μM) in normoxia (n = 33) and ischemia (n = 41); (6) incubation in artificial PL with 1 and 5 mM o-vanadate in normoxia (n = 19) and ischemia (n = 15). After incubation, the cultures were returned to their own culture conditioned medium and were incubated for overnight. Thapsigargin and CPA (Sigma) were dissolved as 100 mM and 10 mM, respectively, stock solutions in dimethylsulfoxide and were stored frozen. Eosin and o-vanadate (Sigma) were stored as aqueous 10 mM and 200 mM, respectively, stock solutions. Aliquots were diluted with artificial PL on the day of use.

24 h after ischemia, the cultures were rinsed with phosphate buffered saline (PBS) and fixed at room temperature in 3.5% paraformaldehyde/0.1 M PBS for 35 min. Then, the fragments were washed two times with PBS and permeabilized with 0.2% triton X-100 in PBS for 30 min. For staining, the fragments were incubated in phalloidin TRIC (tetramethyl rhodamine isothiocyanate, Sigma) at room temperature for 30 min. Phalloidin is a specific marker for cellular F-actin and stains stereocilia and the cuticular plate. The HCs were identified on a Leica DMIL fluorescence microscope. The number of HCs was counted over a distance of 3 times 100 μm in the one IHC row and the three OHC rows at a magnification of x400. Cells were considered missing when there was a gap in the normal geometric array and no stereocilia or cuticular plate were to be seen. Partially damaged hair cells were considered as missing, when more than 50% of the stereocilia and of the cuticular plate were not seen.

The means and standard errors of the mean (SEM) were calculated for all parameters measured. One-way or two-way analysis of variance (ANOVA) was used to compare the HC damage between the experimental groups, the cochlear parts and the IHCs

and OHCs. Additionally, Bonferroni's post hoc test was used for specifically testing the means. $P < 0.05$ was the criterion for significance. All statistical tests and graphs were made using Statistica 7.0 (StatSoft).

All studies were performed in accordance with the German Prevention of Cruelty to Animals Act and permission was obtained from the Berlin Senate Office for Health (T0234/00).

Results

Number of IHCs and OHCs in control and ischemia exposed cultures

Fig. 1 shows representative images of HCs of the organ of Corti under different conditions. The normoxic controls showed a normal regular architecture in the IHC row and in the three OHC rows for up to 48 h of cultivation (**Fig. 1A**). The numbers of IHCs and OHCs amounted to 9.5 ± 0.1 and $12.2 + 0.1/100$ µm, respectively, in each row (**Fig. 2**). In explants exposed to ischemia or eosin, an irregular loss of HCs was observed (**Fig. 1B-D**). Exposure of the cultures to ischemia for 4 h resulted in a significant loss of IHCs and OHCs in the whole organ of Corti counted 24 h after ischemia ($P = 0.0001$ vs. controls). The loss of IHCs amounted to 35-51% and that of OHCs to 15-25% in the apical, middle and basal parts, with the apical parts being less affected in both HC types ($P < 0.01$ vs. middle or basal parts; **Fig. 2**).

Effects of SERCA inhibitors

The SERCA inhibitors thapsigargin (10 nM-10 µM) and CPA (10 and 50 µM), which were tested in this study, had no effect on HC survival in neither the normoxic nor the ischemia-exposed cultures, irrespective of the concentrations used (data not shown).

Effects of PMCA blockers

The PMCA blockers eosin (1.5-10 μM) and o-vanadate (1 and 5 mM) differed in their effects on the HCs in normoxia **(Fig. 3)**. Eosin decreased the HC numbers in a dose-dependent manner. The highest eosin-induced damage was found to occur at a concentration of 10 μM and amounted to about 25% in both HC types as compared to the controls. In contrast, o-vanadate had no effect on the IHC or OHC counts.

Both eosin and o-vanadate aggravated the ischemia-induced HC loss in a dose-dependent manner **(Fig. 4)**. At eosin concentrations of 1.5 μM, no significant effect on either the IHCs or the OHCs was observed. High concentrations (5 μM) of eosin caused 60% of the IHCs to be damaged as compared to ischemia, but they did not affect the OHCs. At a concentration of 10 μM, the eosin-induced damage amounted to about 80% in the IHCs and 50% in the OHCs as determined in the whole organ of Corti. o-vanadate concentrations of 1 mM had no additional damaging effect on the IHCs or OHCs. However, high concentrations (5 mM) induced an additional IHC loss by 45% and an OHC loss by about 50%.

Effects of PMCA blockers on the apical, middle and basal regions of the organ of Corti in ischemia

When the two drugs were analyzed for their separate effects on the IHCs and OHCs in the apical, medial and basal regions, the IHCs' higher vulnerability to eosin in ischemia became obvious in the apical and middle parts **(Fig. 5)**. In contrast, o-vanadate damaged both IHCs and OHCs to similar degrees **(Fig. 6)**. The dose-dependence was similar for the apical, middle and basal regions.

Discussion

The major finding of the present study is that the PMCA blocker eosin induces a dose-dependent HC loss during normoxia and aggravates the ischemia-induced HC damage. The PMCA blocker o-vanadate has no effect on HC survival in normoxia, but enhances ischemia-induced HC loss. In contrast, the SERCA inhibitors thapsigargin and CPA do not affect HC survival in normoxia and ischemia. These data indicate that PMCA is a key enzyme involved in protecting hair cells from ischemia-induced loss.

Role of calcium in ischemia-induced cell death

The primary mechanism thought to be involved in ischemia-induced neuronal death is the massive increase in intracellular Ca^{2+} (Lipton 1999). In general, cytosolic Ca^{2+} may increase as a result of a net influx of Ca^{2+} across the plasma membrane or due to the release of Ca^{2+} from intracellular stores. Specific pathways of ischemia-induced influx of Ca^{2+} into the HCs are not known. It is assumed that the voltage-gated Ca^{2+} channel and NMDA (N-methyl-D-aspartate) receptor-activated Ca^{2+} channels are the main pathways of excessive Ca^{2+} influx, which may lead to HC death (Pujol *et al.* 1990). The roles of SERCA and PMCA in maintaining Ca^{2+} homeostasis following ischemia are presently unknown. In SH-SY5Y neuronal cells, it was shown that in ischemia, endoplasmatic reticulum (ER) Ca^{2+} is released via ryanodine receptor channels, thus contributing to the subsequent cell death (Wang *et al.* 2002). The release of ER Ca^{2+} has two separate consequences: an increase in cytosolic Ca^{2+} levels and a depletion of ER Ca^{2+}, which will disrupt processes like protein folding and processing, i.e., functional activities important to cell viability.

The involvement of PMCAs in ischemic cell damage has been shown by Lehotsky et al. (1999). Transient forebrain ischemia (10 min) and reperfusion was shown to decrease the PMCA immuno-signal. The decrease was ascribed to the loss of the PMCA 1 signal. This group investigated also the possible effects of ischemia and ischemia-reperfusion

injury on ER Ca^{2+} transport (Racay *et al.* 2000). No significant changes of the microsomal Ca^{2+} transport and of the Ca^{2+} ATPase activity were detected during and after ischemia.

Effects of SERCA inhibitors on hair cell survival

Our data show that in the *in vitro* organ of Corti culture, thapsigargin and CPA affect HC survival neither in normoxic nor in ischemic conditions indicating that SERCA has no important role to play in ensuring a certain HC survival rate. This observation is in line with the general functions of the ER (Verkhratsky 2004). It serves as a dynamic Ca^{2+} pool and has important signaling functions in neuronal cells. However, chronic changes in the Ca^{2+} homeostasis are involved in neurodegeneration and neuronal cell death. For example, recently, Bobbin et al. (2003) observed in an *in vivo* guinea pig model that chronic application of thapsigargin (10 μM, 2 weeks) generated OHC loss, while IHCs were occasionally absent. This discrepancy as regards our results may be attributed mainly to the duration of thapsigargin exposure. It is also possible that the influence of SERCA on $[Ca^{2+}]_i$ may be different in different cell types (Yao *et al.* 1999). Our findings to the extent that SERCA inhibitors are not associated with HC death is in agreement with the observation of Martinez-Sanchez *et al.* (2004) who found this cell death to be similar in the presence and absence of CPA following oxygen glucose deprivation in organotypic hippocampal slice cultures.

Effects of PMCA inhibitors on hair cell survival in normoxia and ischemia

The clearly aggravating effect of the two PMCA inhibitors eosin and o-vanadate on HC death supports the assumption that PMCA is a key enzyme for the extrusion of excessive intracellular Ca^{2+} in the HCs (Yamoah *et al.* 1998). The PMCA inhibition is associated with an increase in the $[Ca^{2+}]_i$ in resting cells as shown in neurons from the rat superior cervical ganglion (Wanaverbecq *et al.* 2003). The increase in $[Ca^{2+}]_i$ as caused by

eosin is most probably the reason for HC loss even in normoxic cultures. Unlike eosin, o-vanadate has no effect on HC survival in normoxic conditions. This difference may be associated with specific effects of o-vanadate in addition to PMCA inhibition. For example, sodium o-vanadate is a protein tyrosine phosphatase inhibitor and blocks delayed neuronal death in the CA1 region following ischemic insult (Fukunaga and Kawano 2003).

Our data show that the ischemia-induced HC loss is aggravated by PMCA blockers in an additive or synergistic manner. This led us to the conclusion that PMCA blockers and ischemia act by different mechanisms. This assumption is supported by the finding that caspases cleave and inactivate the PMCA pump in neurons and non-neuronal cells undergoing apoptosis (Schwab *et al.* 2002). The effects of PMCA inhibitors on hair cell survival observed in this paper are in agreement with findings that PMCAs are critical to PC12 cell survival (Garcia and Strehler 1999). Utilizing the model of the Ca^{2+} ionophore A23187 to induce Ca^{2+}-mediated cell death, PMCA depleted PC12 cells expressing about 35% of the PMCA 4 in control cells, were found to be considerably more vulnerable to Ca^{2+}-mediated cell death than control cells.

Differential response of IHCs and OHCs

The IHCs' higher vulnerability to ischemia over that of the OHCs as found in the present study is in agreement with our previous observations (Mazurek *et al.* 2003). Several factors could contribute to the higher vulnerability of IHCs compared to OHCs (Mazurek *et al.* 2003): (1) Ischemia-induced excitotoxicity could participate specifically to the preferred IHC cell death, because glutamate receptors play an important role in signal transduction between IHC and type 1 spiral ganglion (Pujol *et al.* 1990). (2) IHCs seem to produce less glycogen than OHCs, an important substrate under ischemic conditions (Hilding *et al.* 1977). (3) IHCs contain less mitochondria than OHCs, which may regulate the probability of survival after metabolic challenges of HC integrity (Hyde and Rubel 1995). (4) The distribution and function of PMCA isoforms offer an additional explanation for the high IHC vulnerability to ischemia. The main plasma membrane

Ca^{2+} ATPases of mammalian sensory HCs are the isoforms PMCA1 and PMCA2. PMCA1 is located in the HCs' basolateral membrane, whereas PMCA2 is localized exclusively at the apical plasma membrane of the stereocilia of OHCs and IHCs (Grati *et al.* 2006). IHC stereocilia had much less reactivity than those of OHCs. Using a monoclonal antibody to a large cytoplasmic loop of PMCA, a higher reactivity appeared in the cytoplasm of OHCs compared to IHCs (Apicella *et al.* 1997). In the cochlea of 3-5 day old rats, IHCs expressed PMCA1 at moderate levels, and OHCs expressed PMCA2 at high levels (Furuta *et al.* 1998). Assuming that ischemia or eosin and o-vandate inhibit all isoforms to a similar degree, the differential PMCA activity could explain the higher vulnerability of IHCs in the present model.

Another explanation for the differing degrees of IHC and OHC vulnerability to eosin could be their different patterns of regulating $[Ca^{2+}_i]$ (Kennedy 2002). IHCs pump Ca^{2+} out of the cell on an ATP-dependent PMCA, whereas OHCs additionally use the Na^+-Ca^{2+} exchange driven by the Na^+ gradient.

In conclusion, PMCA appears to play a pivotal role in cytoplasmic Ca^{2+} extrusion from the HCs and contributes substantially to the survival of HCs under normoxic and ischemic conditions. In contrast, the Ca^{2+} uptake into the internal stores via SERCA appears to have no or little influence on HC survival.

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Legends to the figures

Fig. 1 Representative images of phalloidin-labelled whole mounts of the rats' organ of Corti under different conditions showing the basal cochlear parts. A- normoxia; B- normoxia and 10 μM eosin; C- ischemia; D- ischemia and eosin (10 μM). Under normoxic conditions, one row of intact inner hair cells (IHCs) and three rows of intact outer hair cells are to be seen. Eosin and ischemia resulted in irregular loss of hair cells, especially of IHCs. Bar 10 μM.

Fig. 2 Mean number (± SEM) of inner and outer hair cells (IHC/OHC)/100 μm per row counted in the apical, middle and basal parts of the organ of Corti in normoxia (n = 19) and ischemia (n = 23) groups (*/**/*** $P < 0.05/0.01/0.001$ vs. normoxia).

Fig. 3 Number of inner and outer hair cells (IHC/OHC; % of controls; mean ± SEM) counted in the normoxia groups with 1.5 μM (n = 15), 5 μM and 10 μM (n = 9 each) eosin or 1 mM (n = 6) and 5 mM (n = 5) o-vanadate (*/** $P < 0.001/0.0001$ vs. controls).

Fig. 4 Number of inner and outer hair cells (IHC/OHC; % of ischemia without drugs; mean ± SEM) determined in the whole organ of Corti in the ischemia groups with 1.5 μM (n = 20), 5 μM (n = 11) and 10 μM (n = 10) eosin or 1 mM (n = 7) and 5 mM (n = 8) o-vanadate (*/** $P < 0.01/0.001$ vs. ischemia without drugs).

Fig. 5 Number of inner and outer hair cells (IHC/OHC; % of ischemia without eosin; mean ± SEM) determined in the apical, middle and basal parts of the organ of Corti in the ischemia groups with 1.5 μM (n = 20), 5 μM (n = 11) and 10 μM (n = 10) eosin.

Fig. 6 Number of inner and outer hair cells (IHC/OHC; % of ischemia without o-vanadate; mean ± SEM) determined in the apical, middle and basal parts of the organ of Corti in the ischemia groups with 1 mM (n = 7) and 5 mM (n = 8) o-vanadate.

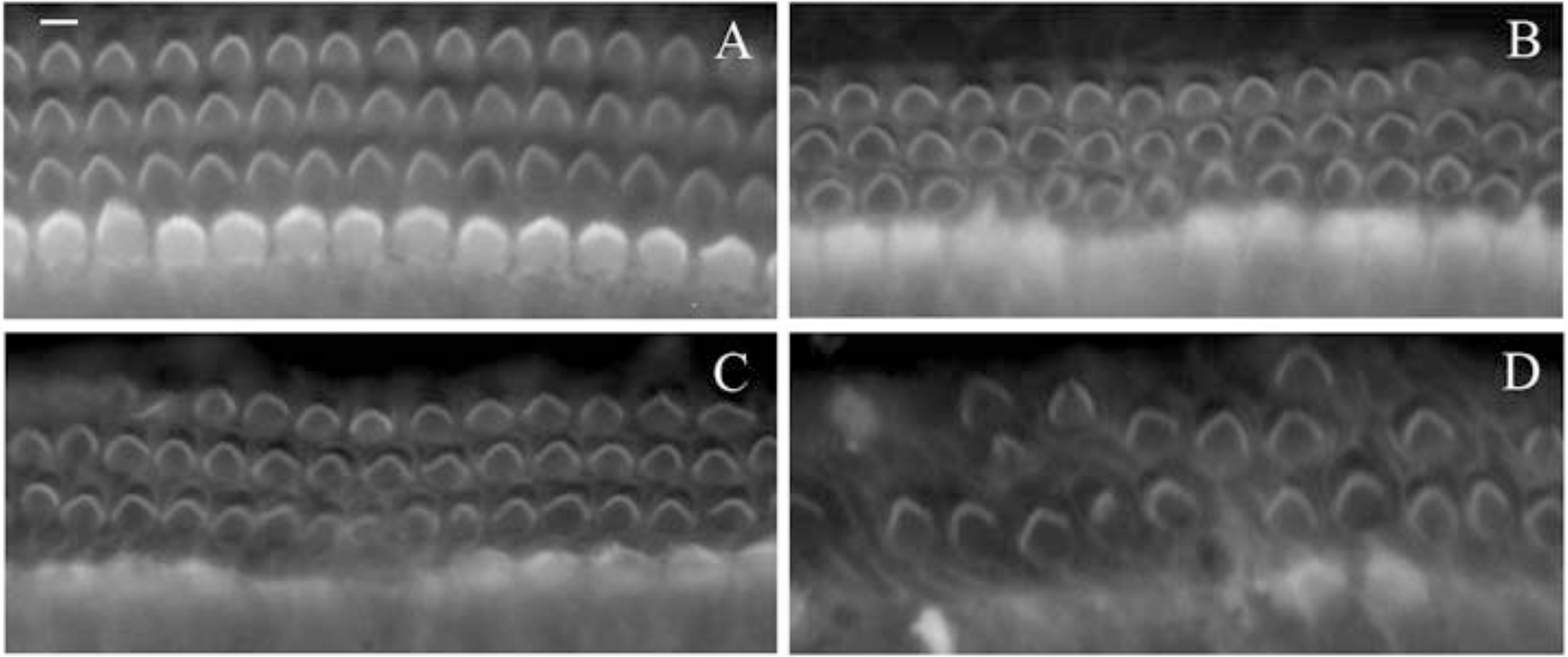

A
B
C
D

Fig. 2

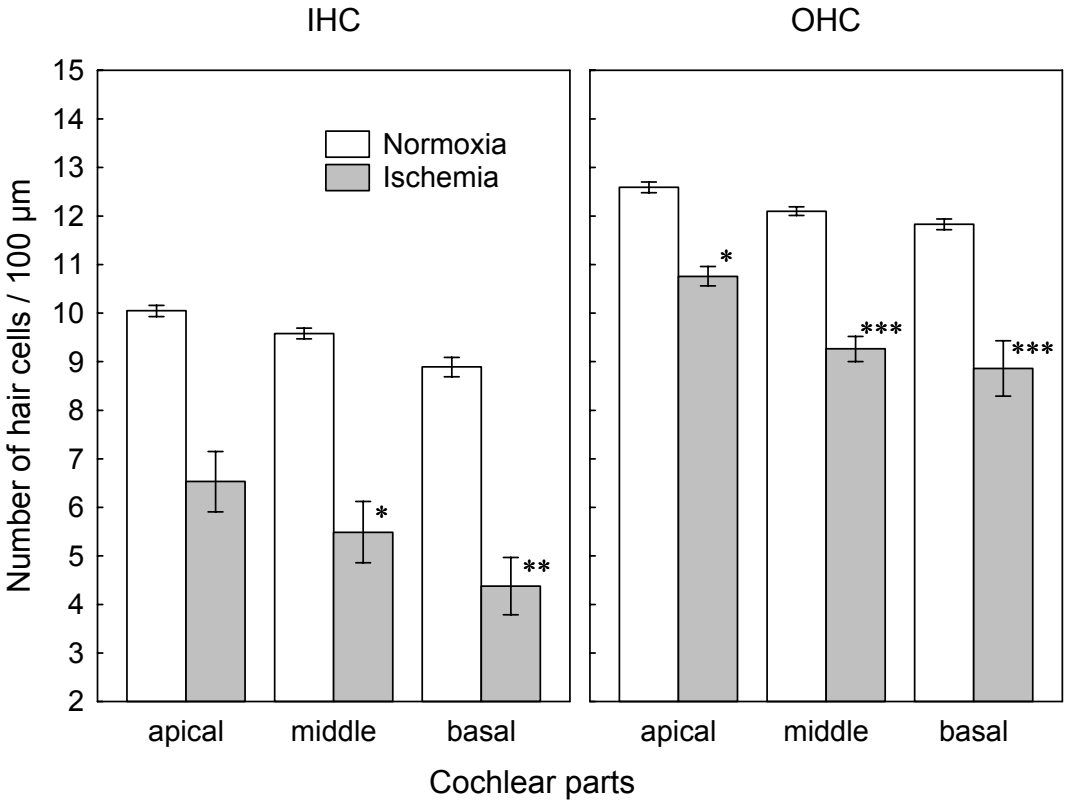

IHC
OHC
Normoxia
Ischemia
Number of hair cells / 100 µm
15
14
13
12
11
10
9
8
7
6
5
4
3
2
*
**
*
***
***
apical
middle
basal
apical
middle
basal
Cochlear parts

Fig. 3

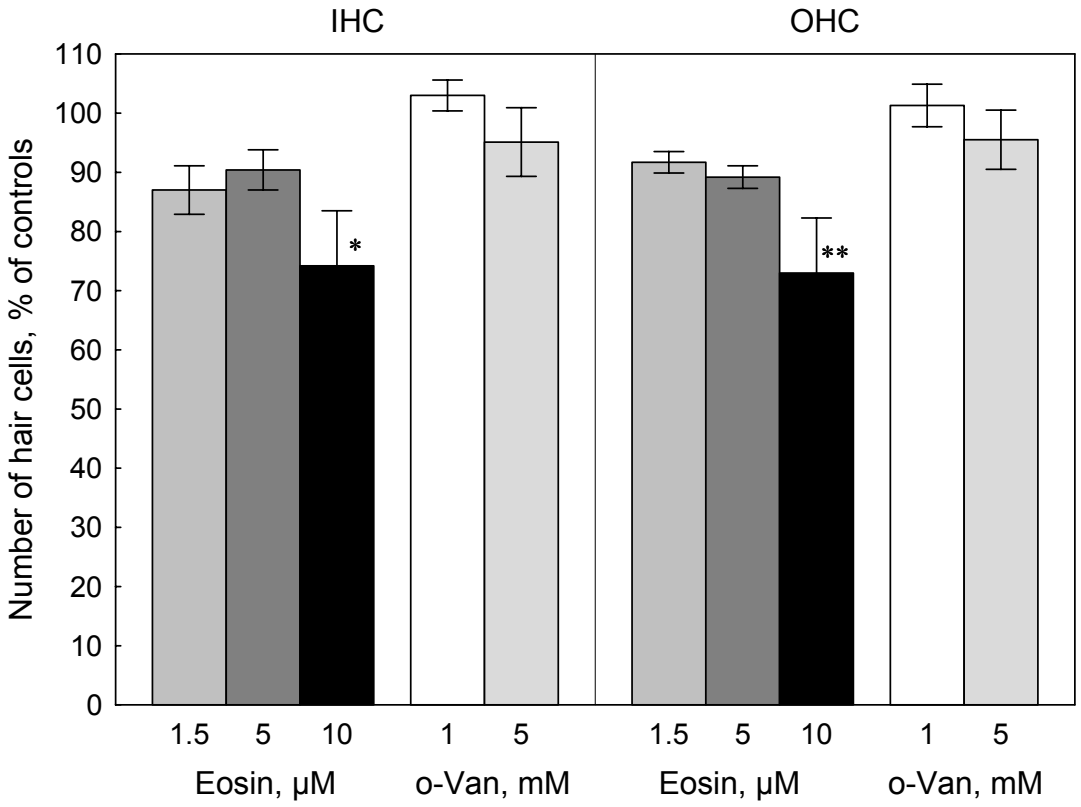

IHC
OHC
Number of hair cells, % of controls
110
100
90
80
70
60
50
40
30
20
10
0
*
**
1.5
5
10
1
5
1.5
5
10
1
5
Eosin, μM
o-Van, mM
Eosin, μM
o-Van, mM

Fig. 4

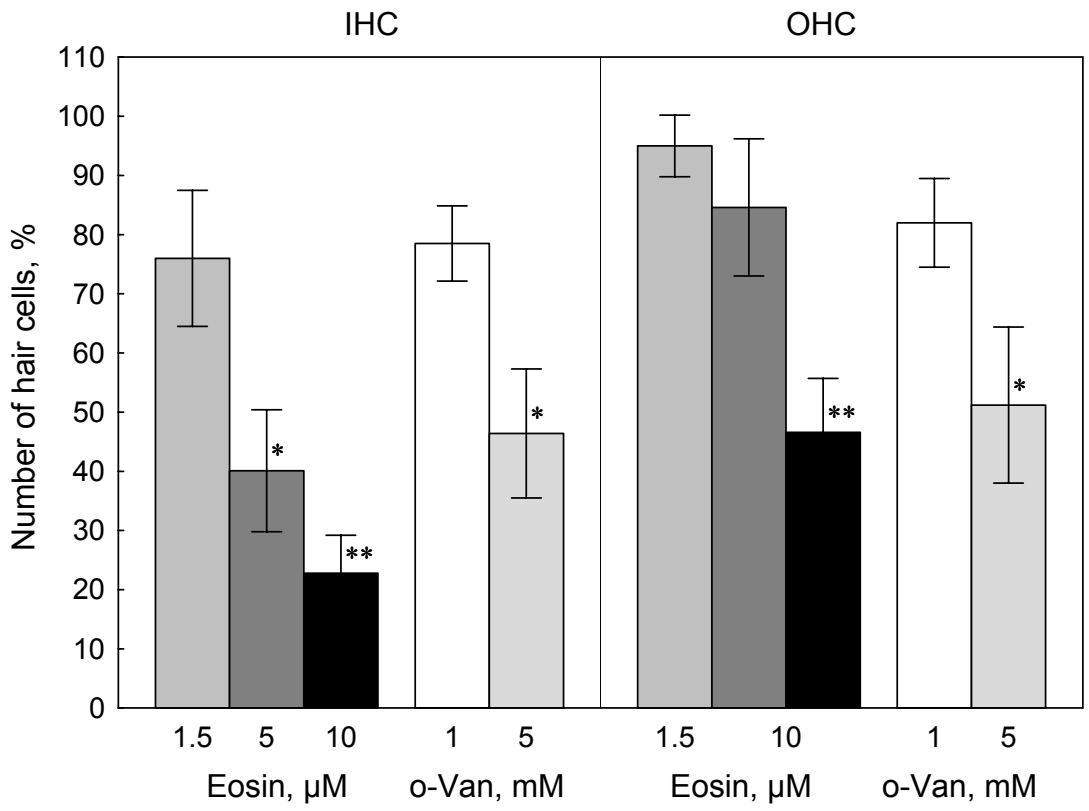

IHC
OHC
Number of hair cells, %
110
100
90
80
70
60
50
40
30
20
10
0
*
**
*
**
*
1.5
5
10
1
5
1.5
10
1
5
Eosin, μM
o-Van, mM
Eosin, μM
o-Van, mM

Fig. 5

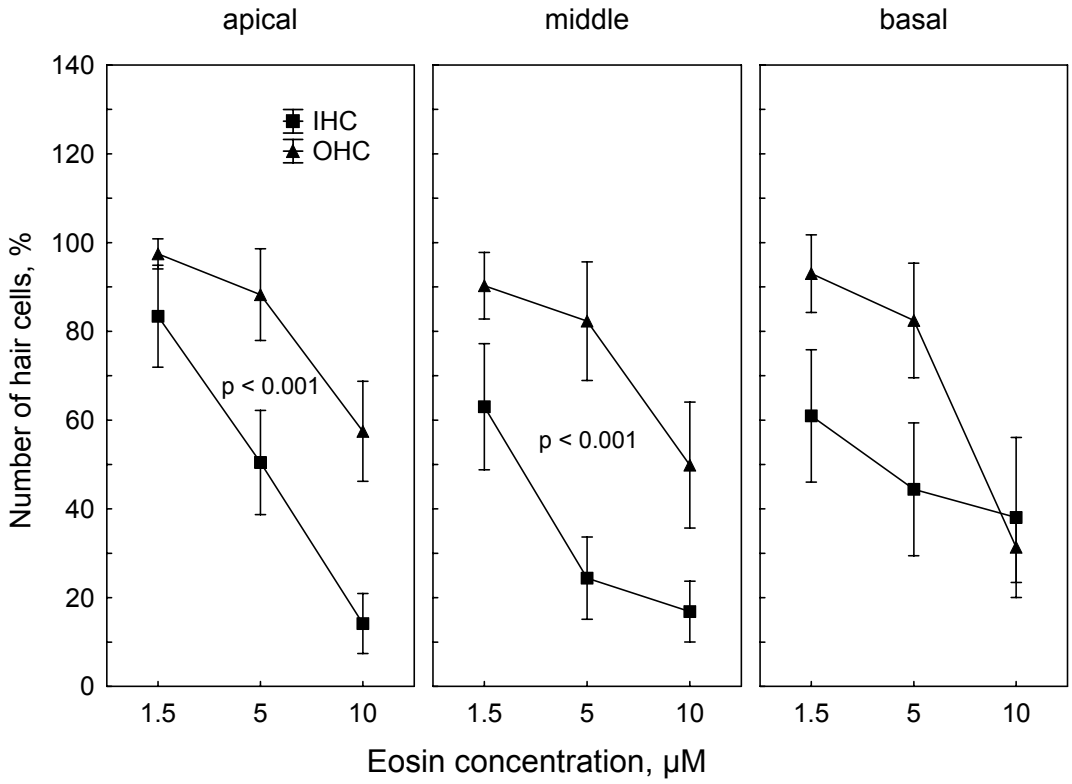
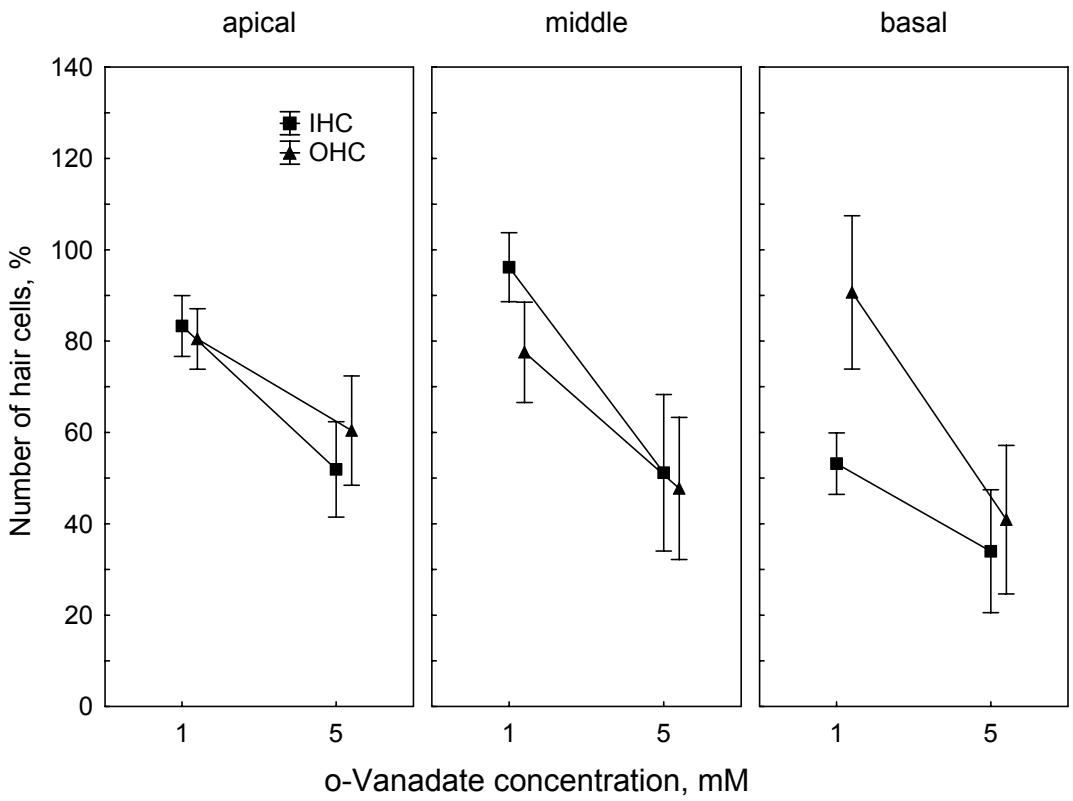

apical
middle
basal
IHC
OHC
p < 0.001
p < 0.001
Number of hair cells, %
0
20
40
60
80
100
120
140
1.5
5
10
Eosin concentration, μM

Fig. 6


apical
middle
basal
IHC
OHC
Number of hair cells, %
140
120
100
80
60
40
20
0
1
5
1
5
1
5
o-Vanadate concentration, mM