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Comparative Examination of Inner Ear in Wild Type and Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)-Deficient Mice

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Abstract Pituitary adenylate cyclase activating polypeptide (PACAP) is a multifunctional neuropeptide with well-known neuroprotective and neurotrophic effects. The involvement of PACAP in sensory processing has also been documented, but little is known about its effects in the auditory system. PACAP and its specific receptor (PAC1) are present in the cochlea and in brain structures involved in auditory pathways. Recently, we have shown that PACAP protects cochlear cells against oxidative stress-induced apoptosis. The endolymphatic Ca^{2+} concentration controlled by Ca^{2+} buffers of the hair cells is essential for the normal hearing processes. In this study we examined the localization of PAC1 receptor and Ca^{2+} buffering proteins (parvalbumin, calretinin, calbindin) in the inner ear of 5-day-old PACAP-deficient mice compared with wild-type mice in order to get a closer insight into the effect of endogenous PACAP in the cochlear function. We did not find differences in the distribution pattern of PAC1 receptors between the two groups, but wild-type animals showed significantly higher PAC1 receptor expression. In contrast, inner and outer hair cells of PACAP-deficient mice showed more pronounced parvalbumin, calbindin, and calretinin immunopositivity compared with wild-type mice. Elevated endolymphatic Ca^{2+} is deleterious for cochlear function, while the high concentration of Ca^{2+} buffers in hair cells may offer protection. The increased immunoreactivity of Ca^{2+} binding proteins in the absence of PACAP provide further evidence the important role of PACAP in the hearing processes.

Keywords Cochlea · Hair cells · PACAP · Knockout · Calcium binding proteins

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide first isolated from the ovine hypothalamus in 1989 based on its adenylate cyclase activating effect (Miyata et al. 1989). It has two forms, PACAP27 and PACAP38, the latter being the dominant

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form in mammals. PACAP is a member of the secretin/glucagon/vasoactive intestinal polypeptide (VIP) family, with 68% similarity to VIP. The PACAP receptors are the VPAC1 and VPAC2 receptors, which bind PACAP and VIP with similar affinity, and the specific PAC1 receptor, which binds PACAP with much higher affinity than VIP (Vaudry et al. 2009). The peptide has wide distribution in the entire body. It is present in the central and peripheral nervous system, in endocrine glands, and also in the cardiovascular, gastrointestinal, and respiratory tracts (Vaudry et al. 2009). Since its discovery numerous studies have shown its neurotrophic and neuroprotective effect both in vitro and in vivo (Arimura et al. 1994; Botia et al. 2007; Ravni et al. 2006; Somogyvari-Vigh and Reglodi 2004). PACAP protects neurons against different toxic insults and it decreases neuronal damage in different animal models of neurodegeneration and ischemic injury (Bourgault et al. 2009, 2011; Brenneman 2007; Dejda et al. 2005, 2008; Nakamachi et al. 2011; Ohtaki et al. 2008; Reglodi et al. 2011).

The involvement of PACAP in sensory processing has also been documented, mainly in the visual and olfactory systems (Atlasz et al. 2010; Han and Lucero 2006). However, little is known about its effects in the auditory system. PACAP immunoreactive elements have been shown in the cochlear nucleus neurons of the brainstem (Hannibal 2002; Kausz et al. 1999), in the spiral ganglion and marginal cells of the stria vascularis with immunohistochemistry and in situ hybridization (Kawano et al. 2001). PACAP precursor protein mRNA and PAC1 receptor mRNA have been described by reverse transcription-polymerase chain reaction in microdissected cochlear lateral wall, organ of Corti and spiral ganglion subfractions (Abu-Hamdan et al. 2006). PACAP and PAC1 receptor play an important role in the modulation of afferent signaling of the organ of Corti (Drescher et al. 2006) and PACAP is present in the olivocochlear neurons of superior olivary complex (Reuss et al. 2009). In line with the well-known neuroprotective effects of PACAP, we have recently shown that PACAP protects cochlear cells against oxidative stress-induced apoptosis (Racz et al. 2010).

In the inner ear, Ca^{2+} buffering is especially important for normal hair cell function. The endolymphatic Ca^{2+} concentration controlled by Ca^{2+} buffers of the hair cells is essential for acoustic transduction and normal hearing processes (Ikeda et al. 2010). Labyrinth destruction causes an elevation in endolymphatic Ca^{2+} concentration, which is important in the disturbance of the cochlear function (Ikeda et al. 2010). Acoustic overstimulation leads to sustained increases in the Ca^{2+} concentration of the outer hair cells, which is toxic to cells (Fridberger et al. 1998). Ca^{2+} buffering proteins (for example: parvalbumin, calbindin, calretinin, calmoduline, calsequestin) have been identified in hair cells of different species, such as in frogs, turtles and mammals (Baird et al. 1997; Hackney et al. 2003; Kerschbaum and Hermann 1993; Pack and Slepecky 1995; Slepecky and Ulfendahl 1993). Hackney et al. (2005) investigated the concentrations of Ca^{2+} buffering proteins in the rat cochlear cells and presumed that high concentration of Ca^{2+} buffers may protect the cells against the toxic effect of Ca^{2+} overload after acoustic overstimulation.

PACAP-deficient mice are used for the examination of endogenous PACAP in different physiological functions and under pathological conditions. It is well known that PACAP-deficient mice have, among others, decreased reproductive function, behavioral abnormalities, memory disturbances, and biochemical alterations (Cummings et al. 2004; Gray et al. 2002; Hashimoto et al. 2001, 2009; Hatanaka et al. 2008; Isaac and Sherwood 2008; Kemeny et al. 2010; Nakata et al. 2004; Tomimoto et al. 2008). The gross morphology of most examined organs does not show significant difference between wild-type and PACAP-deficient mice (Szabadfi et al. 2011; Vaudry et al. 2005). However, numerous studies have shown that PACAP-deficient mice have increased susceptibility for different nervous, renal, and intestinal injuries, supporting the protective effect of endogenous PACAP (Armstrong et al. 2008; Chen et al. 2006; Ferencz et al. 2010; Nakamachi et al. 2010; Ohtaki et al. 2006; Szakaly et al. 2011; Tan et al. 2009; Vaudry et al. 2005). The supposed mechanism for the increased vulnerability of PACAP-deficient mice against various harmful stimuli includes increased apoptosis, inflammatory reactions, and oxidative stress.

Given the known neuroprotective effects of PACAP and its involvement in sensory processing and the importance of Ca^{2+} buffering in the inner ear, the aim of this study was to investigate whether there is any difference in the localization and expression of PAC1-receptor and Ca^{2+} binding proteins (parvalbumin, calretinin, calbindin) in cochlear cells between wild type and PACAP-deficient mice.

Materials and Methods

The generation and maintenance of the knockout mice on the CD1 background have been described previously in detail (Hashimoto et al. 2001, 2009), they were backcrossed for ten generations with the CD1 strain. Wild type ($PACAP^{+/+}$, $n = 9$) and homozygous PACAP-deficient mice ($PACAP^{-/-}$, $n = 9$) were used. Animals were fed and watered ad libitum, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs

(BA02/2000-20/2006). 5-day-old pups were sacrificed with an overdose of anesthetic (Pentobarbital, Nembutal, Sanofi-Phylaxia, Hungary), and the whole heads were immediately fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer for 24 h at room temperature. Tissue was then washed in 0.1 M PB, and cryoprotected in 10% sucrose for 1 h, 20% sucrose in phosphate-buffered saline (PBS) overnight at 4°C. For cryostat sectioning, the heads were embedded in tissue freezing medium (Tissue-Tek, OCT Compound, Sakura Finetech, the Netherlands), cut in a cryostat (Leica, Nussloch, Germany) at 10 µm midsagittal sections. We made approximately 10–12 sections from the cochlea, and we analyzed only those slides in which the modiolus was visibly surrounded by bony spiral canal. We compared only the middle turns of the cochlea. Sections were mounted on chrome–alum–gelatin-coated subbed slides and stored at −20°C until use.

Cryosections were stained with haematoxilin-eosin to identify the different cell types in the organ of Corti and to make a comparison of the inner ear between the wild type and PACAP KO mice. The sections were also processed for immunohistochemical examination. These were rinsed in PBS, permeabilized by incubation for 5 min in 0.1% Triton X-100 in PBS and incubated with 0.1% bovine serum albumin, 1% normal goat serum, and 0.1% Na-azide in PBS for 1 h to minimize nonspecific labeling. Sections were incubated with antibodies directed against PAC1-receptor (PAC1-R) (anti-rabbit; 1:100; Sigma, Hungary); Ca^{2+}-binding proteins: calretinin (anti-mouse; 1:1000; Swant, Switzerland), parvalbumin (anti-mouse; 1:500; Sigma, Hungary), and calbindin (anti-mouse; 1:500; Sigma, Hungary) for overnight at room temperature. After several washes in PBS, sections were incubated for 2 h at 37°C in the dark with Alexa Fluor "568" and "488" secondary antibody (1:1000; Southern Biotech, Hungary). Sections were then washed in PBS and were coverslipped using Fluoromount-G (Southern Biotech, Hungary). For control experiments, primary antisera were omitted and no specific cellular staining was found. Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were taken with the Spot software package. Photographs were further processed with the Adobe Photoshop 7.0 program.

We compared the localization of the different markers stained with immunofluorescence in the cochlear hair cells between wild type and homozygous PACAP KO mice. Immunofluorescence intensity was measured by ImageJ 1.440 software, expression levels were corrected with the tissue background. To compare the intensity of expression in the different groups we standardized the parameters by analyzing every slide from every group at the same time, in the same environment, and the same settings of the program. The statistical analysis made by GraphPadPrism 5.01 program. Results are presented as mean ± SEM, statistical comparisons were made using Student *t* test with Welch's correction and Mann–Whitney test (**$P < 0.001$; ***$P < 0.0001$).

Results

There was no difference in the gross microscopical structure of the inner ear between wild type and PACAP-deficient mice: the different cell types of the organ of Corti could be observed in both groups (Fig. 1a, b). However, immunocytochemical staining revealed clear differences between the cells of the organ of Corti in wild type and PACAP-deficient mice. We examined four markers in the cochlea: PAC1-R (Fig. 1c, d) and three types of Ca^{2+} binding proteins (parvalbumin, calretinin, and calbindin; Fig. 2). In all figures the tectorial membrane displays faint non-specific staining.

PAC1-R expression was found in inner hair cells, outer hair cells, outer phalangeal cells (Deiters' cells), and pillar cells. The distribution of PAC1-R immunopositivity was not different between the two groups, but we observed differences in the intensity of the immunolabeling of inner and outer hair cells of wild type and PACAP KO mice (**$P < 0.001$; Figs. 1c, d and 3a). In wild-type mice the hair cells and outer phalangeal cells showed more intense immunolabeling compared to PACAP-deficient mice (Figs. 1c, d and 3a).

Examining Ca^{2+} binding proteins, the hair cells showed weak parvalbumin, calretinin and calbindin-immunopositivity in wild-type mice (Figs. 2a, c, e and 3b–d). In contrast, both inner and outer hair cells showed significantly stronger immunopositivity to parvalbumin in the PACAP-deficient mice, especially their hair bundles (stereocilia). The immunostaining was more intense in the entire cell bodies, compared to wild-type cochleas (Figs. 2a, b and 3b). Inner and outer hair cells of PACAP KO mice also showed more pronounced calretinin immunopositivity compared to wild-type mice (Figs. 2c, d and 3c). In PACAP-deficient mice calretinin was present, accumulated in the hair bundles of the hair cells and less intensely stained in their cell bodies (Figs. 2d and 3c). A weaker calbindin labeling was found in wild-type mice compared with parvalbumin or calbindin (Fig. 2e). In contrast, a more pronounced calbindin expression was observed in PACAP-deficient mice (Figs. 2f and 3d).

Immunofluorescence intensity was measured and compared in the inner and outer hair cells of wild type and PACAP-deficient mice. All of the observed differences between the wild type and PACAP KO animals were significant according to the immunofluorescence intensity measurements (Fig. 3a–d).

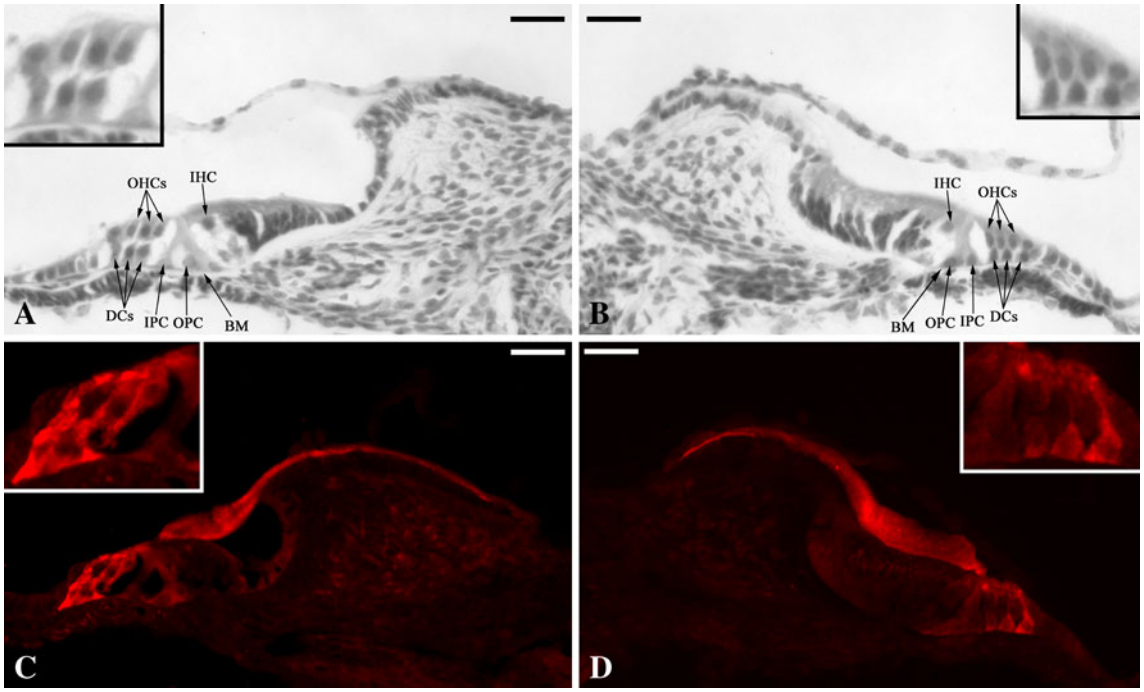

Fig. 1 Histological analysis and the localization of PAC1 receptor immunoreactivity in the organ of Corti in wild type (**a** and **c**) and PACAP-deficient mice (**b** and **d**). No differences were observed in the structure and cell types of the organ of Corti between the two groups (**a** and **b**). PAC1 receptor immunolabeling is found in the OPCs and less intensely in the IHCs and OHCs. In wild-type mice (**c**) OHCs and OPCs show more intense immunolabelling compared to PACAP-deficient mice (**d**). *Scale bar* 20 μm. *OHC* outer hair cell, *IHC* inner hair cell, *DC* Deiter's cell, *IPC* inner phalangeal cell, *OPC* outer phalangeal cell, *BM* basal membrane

Discussion

In this study, we provided evidence for the altered expression of PAC1-R and Ca^{2+} binding proteins in the inner ear of PACAP-deficient mice. Although the gross microscopical structure of the inner ear did not show alteration in PACAP-deficient mice, comparative immunohistological examination revealed significant differences in the expression of PAC1-R and Ca^{2+} binding proteins (parvalbumin, calretinin, and calbindin) of the inner and outer hair cells between wild type and PACAP-deficient animals.

We first investigated the expression of PAC1-R in the organ of Corti. We found that both inner and outer hair cells showed PAC1-R expression in wild-type mice and also demonstrated PAC1-R positivity in the outer phalangeal cells (Deiters' cells). Some studies have already described the localization of PAC1-R in the inner ear. Abu-Hamdan et al. (2006) showed two splice variants of PAC1-R in rat microdissected cochlear subfraction. Similarly to our observations, Drescher et al. (2006) found PAC1-R immunopositivity in hair cells and Deiters' cells. In our study we demonstrated that both hair cell types and Deiters' cells of PACAP-deficient mice expressed PAC1-R, but this expression was significantly lower in PACAP-deficient mice compared to their wild-type mates. The regulation of the PACAP-R subtypes in the central and peripheral nervous system is not well understood, but Girard et al. (2006) have also found a developmental delay in the expression of all three receptor subtypes in the brains of PACAP- and VIP-deficient animals compared to their wild-type counterparts.

Next we investigated the expression of Ca^{2+} binding proteins in wild type and PACAP-deficient mice. We found that inner and outer hair cells showed parvalbumin, calretinin, and calbindin-immunopositivity in the cochlea of the wild-type mice on postnatal day 5 (P5). This age was chosen based on studies describing the developmental changes of Ca^{2+} binding proteins, providing evidence that the proteins investigated in our study are all present at this age. Given the importance of Ca^{2+} buffering in the inner ear, numerous studies have investigated the presence and of Ca^{2+} binding proteins in the cochlear hair cells in different species (Baird et al. 1997; Dechesne et al. 1991, 1994; Hackney et al. 2003; Kerschbaum and Hermann 1993; Pack and Slepecky 1995; Schwaller 2010; Slepecky and Ulfendahl 1993; Soto-Prior et al. 1995; Yang et al. 2004). Ca^{2+} binding proteins (calretinin, calbindin, parvalbumin) play important roles in buffering cytoplasmic Ca^{2+} transients. Calretinin immunoreactivity appears in the inner hair cells between embryonic day 19—P0, while outer hair cells express it on P1. All the inner and outer hair cells are

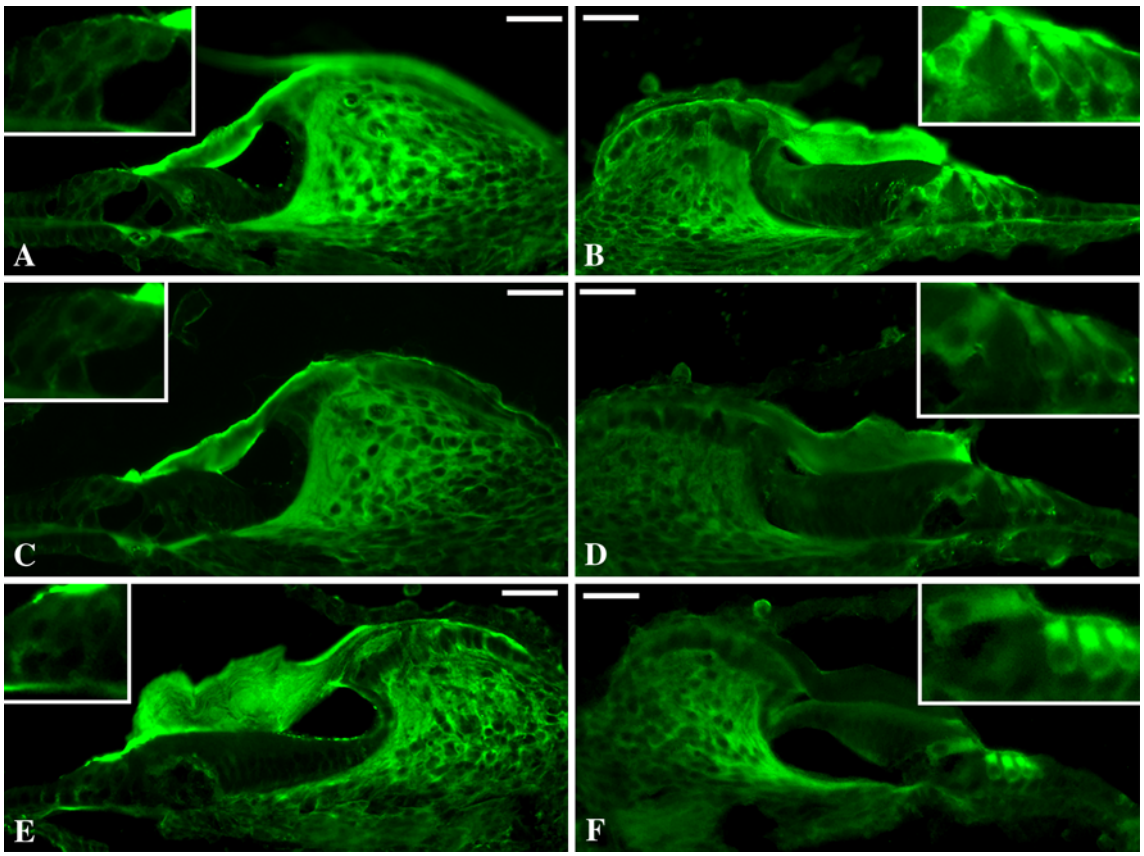

Fig. 2 Cryosections of the mice cochlea show parvalbumin (**a**–**b**), calretinin (**c**–**d**), and calbindin (**e**–**f**) immunostaining. More intense parvalbumin-, calretinin-, and calbindin-immunoreactivity is seen in the cell bodies of inner and outer hair cells and also their stereocilia in PACAP KO mice (**b**, **d**, and **f**) compared to wild-type mice (**a**, **c**, and **e**). *Scale bar* 20 μm

positive on P4, but from P10 the immunopositivity of the outer hair cells starts to disappear (Dechesne et al. 1994). Examinations of adult rats, guinea pigs, and Mongolian gerbil cochlea show a distribution of calretinin similar to mice (Dechesne et al. 1991; Pack and Slepecky 1995). Parvalbumin has two isoforms, the α- and β-parvalbumin (Pauls et al. 1996). In the rat cochlea β-parvalbumin expression begins between P2 and P4, and peaks on P10, while α-parvalbumin expression starts before birth and remains high in the adult period (Hackney et al. 2005; Yang et al. 2004). Sakaguchi et al. (1998) found similar results in gerbil and mouse inner ears. In our experiments we used a parvalbumin antibody labelling all isoforms and demonstrated immunopositivity in both hair cell types. Calbindin is found in the cytoplasm of both inner and outer hair cells on P7 rats and declines in both cell types during maturation being absent from inner hair cells by P26 (Hackney et al. 2005).

In addition to the different ototoxic agents, acoustic overstimulation is one of the main causes of deafness resulting from sustained increases in the Ca^{2+} concentration of the hair cells (Ikeda et al. 1988; Li et al. 2011; Lendvai et al. 2011). It is well known that increased intracellular Ca^{2+} concentration is toxic to cells (Orrenius et al. 1992; Trump and Berezesky 1996), through activation of Ca^{2+}-dependent enzymes. The Ca^{2+}-induced cell toxicity can be blocked if the Ca^{2+} increase is prevented (Gilbert et al. 1995). It has been demonstrated that acoustic trauma causes increase in Ca^{2+} concentration in the endolymph, generating an elevated entry through the transducer channels of the cells (Fridberger et al. 1998; Ikeda et al. 1988). The endolymphatic Ca^{2+} concentration is also elevated after vestibular labyrinth destruction (Ikeda et al. 2010) and surgically induced endolymphatic hydrops, which was induced by ablation of endolymphatic duct (Salt and DeMott 1994). In our study we showed that the expression of all examined Ca^{2+} binding proteins (parvalbumin, calretinin, calbindin) were elevated in the hair cells of PACAP-deficient mice. The increased expression of Ca^{2+} binding proteins has been shown after sound stimulation in the inferior colliculus (Idrizbegovic et al. 1999) and in the cochlear nucleus of mice (Idrizbegovic et al. 1998). Examination of the C57 mice, as a suitable model for progressive age-related sensorineural hearing loss called presbyacusis, has revealed that degenerative changes in the auditory system can modulate neuronal

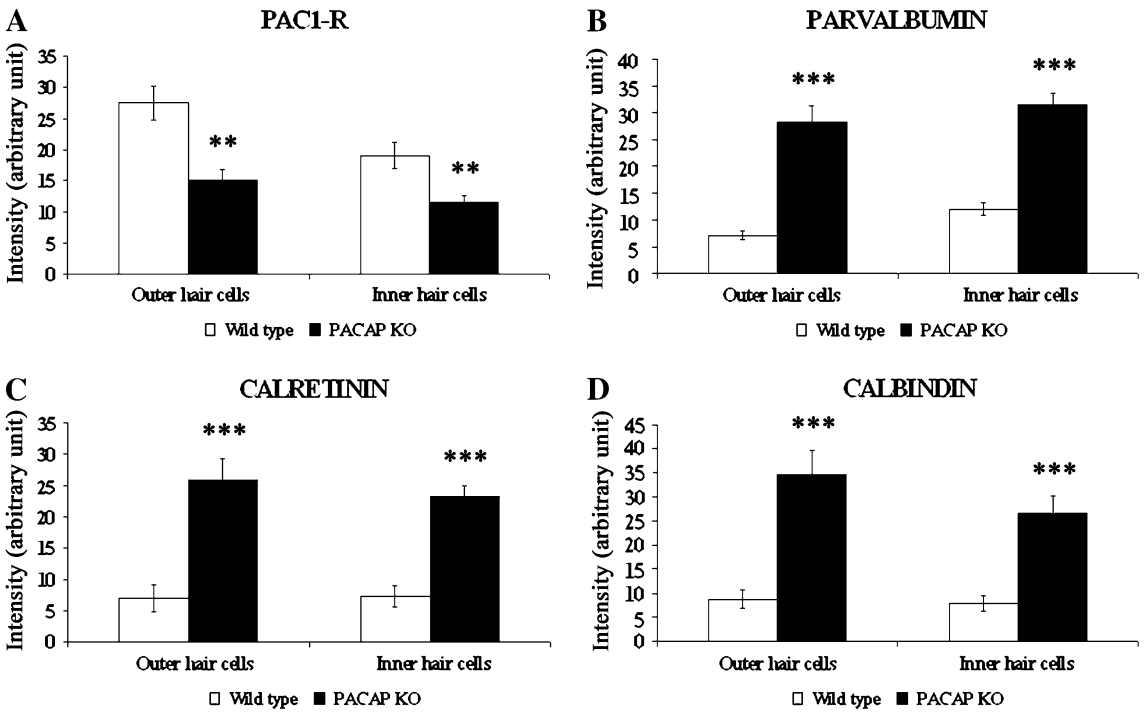

Fig. 3 Statistical comparison of immunofluorescence intensity between the hair cells of wild type- and PACAP-deficient mice (**a**–**d**). PAC1 receptor expression (**a**) was significantly decreased in both inner and outer hair cells of PACAP-deficient mice compared to the wild-type group. In contrast, staining for all three calcium binding proteins (parvalbumin, **b**; calretinin, **c**, and calbindin, **d**) was significantly increased in hair cells of PACAP-deficient group. $^{**}P < 0.001$ wild type versus PACAP-deficient mice; $^{***}P < 0.0001$ wild type versus PACAP-deficient mice

homeostasis by increasing Ca^{2+} binding proteins in the posteroventral and dorsal cochlear neurons during aging. Increase in parvalbumin and calretinin immunoreactivity has been observed in both nuclei in 1–30-month-old mice (Idrizbegovic et al. 2003). Similar results have been found in 24-month-old BALB/c mice, a model of sensorineuronal hearing loss, suggesting an important role of Ca^{2+} binding proteins in the protection against age-induced Ca^{2+} toxicity (Idrizbegovic et al. 2006). The exact cause of elevated Ca^{2+} binding protein expression in the hair cells of PACAP-deficient mice is unknown. A protective role for Ca^{2+} binding proteins in certain neuronal population has been postulated earlier (Heizmann 1992). The neuronal calbindin content may protect neurons from Ca^{2+} toxicity: for example in substantia nigra lesion the majority of degenerating cells do not contain calbindin, but the small percentage of surviving cells are calbindin positive (Iacopino et al. 1992). The striatal neurons poor in calbindin also show greater vulnerability to ischemia and Huntington's disease (Figueredo-Cardenas et al. 1998). The loss of calretinin and parvalbumin expression or the loss of calretinin- and parvalbumin-containing cells in the hippocampus may also play a role in the progressive nature epilepsy after electrically induced status epilepticus (van Vliet et al. 2004).

PACAP-deficient mice are also more vulnerable to stressors of the nervous system and harmful stimuli of the peripheral organs such as kidney and small intestine (Armstrong et al. 2008; Chen et al. 2006; Ferencz et al. 2010; Nakamachi et al. 2010; Ohtaki et al. 2006; Szakaly et al. 2011; Tan et al. 2009; Vaudry et al. 2005). PACAP has well-known neurotrophic and neuroprotective effects. PACAP protects neurons against different toxic agents, such as 6-hydroxydopamine, ethanol, oxidative stress, and anisomycin (Reglodi et al. 2011; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009), but also non-neuronal cells, such as endothelial cells, cardiomyocytes, and kidney cells (Gasz et al. 2006; Horvath et al. 2010; Racz et al. 2007). Endogenous PACAP and its receptors are upregulated after focal and global ischemia (Gillardon et al. 1998; Riek-Burchardt et al. 2010; Shin et al. 2001; Stumm et al. 2007), traumatic brain injury and different nerve injuries (Larsen et al. 1997; Moller et al. 1997; Skoglosa et al. 1999; Zhang et al. 1996, 1998; Zhou et al. 1999). Vaudry et al. (2005) were the first to show that granule cells from PACAP-deficient mice react to cellular stressors such as ethanol and oxidative stress with higher sensitivity. Numerous experiments have proven the protective effect of exogenous PACAP treatment in different models of ischemia and neurodegenerative disorders (Dohi et al. 2002; Ohtaki et al. 2008; Reglodi et al. 2002, 2011; Somogyvari-Vigh and Reglodi 2004; Tamas et al. 2002). Both homozygous and heterozygous PACAP-deficient mice have higher infarct volume and neurological

deficits in models of stroke (Chen et al. 2006; Nakamachi et al. 2010; Ohtaki et al. 2006). There are numerous data about the retinoprotective effects of PACAP after ischemic injury (Atlasz et al. 2007, 2010; Szabadfi et al. 2010). Recently, our research group has shown that PACAP-deficient mice have more severe retinal degeneration after bilateral carotid artery occlusion followed by 2-week reperfusion period that can be attenuated by exogenous PACAP treatment (Szabadfi et al. 2011).

Recently, we have proven the protective effect of PACAP in chicken cochlear cell culture against oxidative stress. PACAP increased the cell survival and decreased the activation of caspase-3 in H_2O_2-induced oxidative stress (Racz et al. 2010). Although ototoxicity has not yet been investigated in PACAP-deficient mice, the otoprotective effect of PACAP, and the higher vulnerability of mice lacking PACAP to different stressors suggest that PACAP-deficient mice would also show increased sensitivity to hair cell toxicity. Our present observation showing increased expression of Ca^{2+} binding proteins in the inner ear of PACAP-deficient mice supports this hypothesis, given the protective role of Ca^{2+} binding proteins against the toxicity induced by elevated intracellular Ca^{2+}. Although PACAP itself is able to increase intracellular Ca^{2+} through phospholipase C signaling (Vaudry et al. 2009), it has also been described that the glutamate-induced pathological increase of intracellular Ca^{2+} concentration can be inhibited by PACAP and thereby neuroprotection can be achieved in hippocampal neurons (Dong et al. 2000). In summary, our study presents data, for the first time, on the inner ear structure of PACAP-deficient mice and suggests that PACAP deficiency may mean higher vulnerability of cochlear cells against toxic agents. As an endogenous compensatory mechanism, a higher Ca^{2+} binding protein concentration can be observed in the cells of the inner ear.

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