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► To cite this version:

Sabine Ladrech, Jing Wang, Hassan Boukhaddaoui, Jean-Luc Puel, Michel Eybalin, et al.. Differential expression of PKC beta II in the rat organ of Corti.. European Journal of Neuroscience, 2007, 26 (10), pp.2922-30. 10.1111/j.1460-9568.2007.05916.x . inserm-00182838

HAL Id: inserm-00182838

<https://inserm.hal.science/inserm-00182838>

Submitted on 23 Jun 2014

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Receiving Editor: C. Mulle, Bordeaux, France

Differential expression of PKC beta II in the rat organ of Corti

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Running title: PKC beta II expression in the rat organ of Corti

27 pages, 3 figures, 1 table

Whole manuscript: 7104 words; Abstract: 241 words; Introduction: 489 words

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Key words: cochlea, efferent systems, synapses, development

ABSTRACT

To investigate a possible involvement of protein kinase C (PKC) in cochlear efferent neurotransmission, we studied the expression of the calcium dependant PKC beta II isoform in the rat organ of Corti at different postnatal ages using immunofluorescence and immunoelectron microscopy. We found evidence of PKC beta II as early as postnatal day (PND) 5 in efferent axons running in the inner spiral bundle and in Hensen cells. At PND 8, we also found PKC beta II in efferents targeting outer hair cells (OHCs) and a slight detection at the synaptic pole in the first row of the basal and middle cochlear turns. At PND 12, PKC beta II expression declined in the efferent fibres contacting OHCs whereas expression was concentrated at the postsynaptic membrane, from the basal and middle turns. The adult-like pattern of PKC beta II distribution was observed at PND 20. Throughout the cochlea, we found PKC beta II expression in the Hensen cells, nonsensory cells involved in potassium re-cycling, and lateral efferent terminals of the inner spiral bundle. In addition, we observed expression in OHCs at the postsynaptic membrane facing the endings of the medial efferent system, with the exception of some OHCs located in the most apical region of the cochlea. These data therefore suggest an involvement of PKC beta II in both cochlear efferent neurotransmission and ion homeostasis. Among other functions, PKC beta II could play a role in the efferent control of OHC activity.

INTRODUCTION

Two types of sensory receptor within the mammalian cochlea, the inner (IHCs) and the outer (OHCs) hair cells, receive distinct efferent supplies from two separate systems originating in the brainstem superior olivary complex: the lateral (LOC) and the medial olivocochlear (MOC) respectively (for review see Guinan, 2006). The LOC axons form “en-passant” axodendritic synapses on radial afferent fibres synapsing with the IHCs, whereas MOC axons form large axosomatic synapses with the OHCs (for review, see Pujol et al., 1998).

Both efferent cochlear systems form a feedback loop, with previously proposed implication in cochlear protection against noise trauma (d’Aldin et al., 1995; Ruel et al., 2001; Maison et al. 2002; Luebke & Foster, 2002) and ischemia induced excitotoxicity (d’Aldin et al., 1995); both diseases resulting in a permanent threshold shift that may induce tinnitus (for reviews see Puel et al., 2002; Lustig, 2006). The MOC regulates cochlear active mechanisms by modulating OHC motile activities (for review, see Geleoc & Holt, 2003) and mediates the protection of OHCs through cholinergic activation of alpha9-alpha10 nicotinic receptors (Maison et al., 2002; Luebke & Foster, 2002). In addition, the MOC may be implicated in the particular vulnerability of the OHCs to aminoglycoside antibiotics (Brown & Daigneault, 1973). The LOC modulates the auditory message sent to the brain by regulating the dendritic activity of spiral ganglion neurons connected to the IHCs, preventing excitotoxicity via tonic dopamine inhibition (Ruel et al., 2001, 2007). The identification of the molecular players involved in cochlear efferent neurotransmission is thus needed in order to define experimental therapeutic targets for major hearing dysfunctions.

Several phosphorylation pathways involving calcium/calmodulin (Dulon et al., 1990; Coling et al., 1998), cyclic GMP (Szonyi et al., 1999) or RhoA and ROCK (Zhang et al., 2003) dependant mechanisms, are thought to mediate the regulation of OHC slow and fast motility. Protein kinase C (PKC) is believed to be involved in repair (Lerner-Natoli et al., 1997) and protection (Lallemend et

al., 2005) of spiral ganglion neurons in various stress conditions. Moreover, PKC activation has been shown to protect OHCs from antibiotic ototoxicity (Chung et al., 2006).

The ubiquitously expressed protein kinase C (PKC) family of kinases have key roles in regulating major cellular activities including synaptic transmission in neurons (for reviews, see Nicholls, 1997 and Dempsey et al., 2000). There are at least 12 PKC isoforms classified into three subfamilies according to the structure of the N-terminal regulatory domain which determines their sensitivity to the second messengers Ca^{2+} and diacylglycerol (for reviews, see Parker & Murray-Rust, 2004 and Poole et al., 2004). The identification of specific PKC activators and inhibitors makes PKC an attractive target in molecular therapeutics (for cancer therapeutics, see: Serova et al., 2006).

Since the conventional Ca^{2+} and diacylglycerol dependant PKC beta isoform has been shown to mediate neurotransmitter release and receptor phosphorylation (among others: Yang et al., 2002; Pacheco et al., 2003), we investigated the possible PKC beta II involvement in cochlear efferent neurotransmission.

MATERIAL AND METHODS

Experiments were carried out in accordance with the animal welfare guidelines of the Institut National de la Santé et de la Recherche Médicale and approved by the French Ministère de l'Agriculture et de la Forêt. We used a total of 31 Wistar rats aged from postnatal day (PND) 5 to PND 60, with 19 normal rats employed for immunocytochemistry (14 for immunofluorescent labelling and 5 for immunoelectron microscopy) and 6 adult (35 day old) and 6 juvenile (12 day old) sacrificed for western blotting.

Antibodies

In addition to the anti-PKC antibody against the isoenzyme beta II (rabbit polyclonal, sc 210, lots L1602 or H1805, Santa Cruz, dilution: 1/200), four other antibodies were employed: two against calbindin (mouse monoclonal, C9848, Sigma, dilution: 1/1000; and goat polyclonal, sc210, Santa Cruz, dilution: 1/50) to recognize the hair cells (Dechesne & Thomasset, 1988), one against neurofilaments, NF 200 kD (mouse monoclonal, 1178709, Boehringer, dilution: 1/100) to target nerve fibres (Romand and Romand, 1985), one against synaptophysin (mouse monoclonal, clone 5vp-38, Sigma, Saint-Louis, MO, USA, dilution: 1/1000) and one against cholineacetyltransferase (ChAT) (goat polyclonal, Chemicon, Temecula, CA, USA, dilution: 1/100) to specifically identify the efferent terminals (Eybalin & Pujol, 1987; Gil-Loyzaga & Pujol, 1988). The secondary antibodies used were a donkey antirabbit IgGs conjugated to Alexa 488, 1/1000 (Molecular Probes, Eugene, OR, USA), a donkey antirabbit IgGs conjugated to Alexa 568, 1/1500 (Molecular Probes), a donkey antimouse IgGs conjugated to Alexa 568, 1/1000 (Molecular Probes) and a donkey antigoat IgGs conjugated to Alexa 647, 1/200 (Molecular Probes).

Immunofluorescent labelling

After decapitation under deep anesthesia (Pentobarbital 50 mg/kg), the cochleae were removed from the temporal bone, perfused with a solution of 4% paraformaldehyde in 0.1M - pH 7.3

phosphate-buffered saline and post-fixed in the same fixative for 3 hours at room temperature. Cochleae were then rinsed in the phosphate buffer and the surrounding bony capsule dissected.

Whole-mount surface preparations

After dissection of the bony capsule, labelling procedures (see below) were performed on the whole cochlear spiral and the basal (5mm long), middle (3mm long) and apical (1mm long) cochlear turns were then separated from the modiolus and mounted in Vectashield (VH1000, Vector).

Cryostat sections

The samples (including the stria vascularis, the organ of Corti and the modiolus) were soaked overnight in 0.1 M phosphate buffer containing 20% sucrose at 4°C and frozen at – 80°C in OCT. Serial 12-µm thick mid-modiolar sections were cut on a cryostat and stored at -20°C until use.

Labelling procedures

Double or triple labelling was performed on either whole mount preparations or cryostat sections. Samples were incubated for 30 min at room temperature in 30 % normal donkey serum (NDS) in PBS containing 0.3% Triton X-100. These were then incubated for 24 hours at 4°C in the primary antibodies containing 0.1% Triton X-100 and 1% of normal donkey serum. After four rinses of 15 min, the organ of Corti was incubated for 2 hours at room temperature in PBS containing secondary antibodies. After a final rinse (4x15min), samples were mounted in Vectashield (VH 1000, Vector, Burlingam, CA, USA). Negative controls were carried out by omitting the primary antibodies in the first incubation step. No fluorescence was observed in these conditions.

Confocal microscopy

Cryostat sections and whole-mount surface preparations were observed under a MRC 1024 laser scanning confocal microscope (BioRad) equipped with a 15-mW krypton/argon laser. Optical sections (0.2 to 1 µm thick) were recorded using Kalman-averaging of four consecutive scans to reduce noise. The fluorescence emission was collected sequentially using the 488 nm, 568 nm and

647 nm lines for Alexa 488, 568 and 647 respectively. The computer superimposed onto single planes Alexa fluorescence of 5 to 30 optical sections. Brightness and contrast of the final image was optimized using Confocal Assistant and Adobe Photoshop. The 3D structure was reconstructed from confocal images using AMARIS software (version Bitplane AG).

Immunoelectron microscopy

After decapitation under deep anaesthesia (Pentobarbital 50 mg/kg), the cochleae were removed from the temporal bone, perfused with a solution of 4% paraformaldehyde in 0.1M - pH 7.3 phosphate-buffered saline, post-fixed in the same fixative for 1 hour at room temperature, washed several times in the phosphate buffer (2X10 mn) and then dissected from the otic capsule.

We used a similar pre-embedding immunocytochemical procedure to one previously used (Eybalin et al., 1993). Briefly, after an *in toto* incubation with the anti-PKC beta 2 antibody diluted 1/200 in PBS-1% normal goat serum (24 hours at 4°C), the cochleae were incubated for 1h with HRP-conjugated goat Fab fragments against anti-rabbit IgGs (Abcys, Paris, France). After cytochemical detection with either DAB (Sigma) or VIP (Vector), the specimens were post-fixed for 1 hour in 2% osmium tetroxide and embedded in epon resin. Transverse ultra-thin sections of the organs of Corti were then cut and observed uncontrasted at 50 kV using a HITACHI-H7100 transmission electron microscope.

Negative controls were carried out as before by omitting the primary antibody in the first incubation step. Neither DAB nor VIP deposits could be observed in these conditions.

Western blot analysis

The specificity of the anti-PKC beta II antibody was assessed using Western blotting in the cochlear tissue. Two tissue lysates from 4 cochleae taken from either adult rats (35 day-old) or juvenile rats (12 day-old) were separated by SDS-PAGE and transblotted onto nylon membranes. Blots were incubated overnight at 4°C with rabbit polyclonal antibody to PKC beta II (1:400; sc 210, lots

L1602 or H1805, Santa Cruz) and mouse monoclonal antibody to β -actin (1:500, Sigma), then incubated with alkaline phosphatase-conjugated secondary antibodies (Sigma). Protein-antibody complexes were revealed with BCIP/NBT-Purple Liquid Substrate (Sigma). The experiment was repeated 3 times.

RESULTS

Western blotting

Of the three experiments conducted in both adult and juvenile rat cochleae, only one immunoreactive band was evident using the PKC beta II antibody. This band localized at 80 kD, the molecular weight of the PKC beta II isoenzyme (Figure 1).

Immunofluorescence

Expression pattern of PKC beta II in the adult cochlea

In mid-modiolar sections of the organ of Corti, PKC beta II appeared to be expressed in three different structures: 1) nerve fibres of the inner spiral bundle, 2) the basal (synaptic) pole of the OHCs or 3) the nonsensory epithelial Hensen cells (Figure 2a).

In the inner spiral bundle, the expression of PKC $_{\beta II}$ co-localized with that of synaptophysin all along the cochlear spiral (Figure 2b) indicating the presence of PKC beta II within the efferent terminals of the LOC. We found no PKC beta II immunoreactivity within either the IHCs or afferent nerve fibres unlabelled for synaptophysin.

In the basal and middle cochlear turns, all OHCs expressed PKC beta II. Such expression appeared as disks underlining portions of the basolateral wall of the OHCs, facing MOC efferent endings labelled for either neurofilaments (Figure 2c), synaptophysin (Figure 2d-f) or ChAT (Figure 2g). Each OHC generally presented several disks (between two and four), although a few OHCs showed a single large disk (Figure 2d) possibly formed by several smaller disks (unresolved at this magnification) corresponding to distinct efferent synaptic sites.

In the most apical cochlear region (the last 300 μ m), a number of OHCs generally lacked PKC beta II expression (Figures 2h and 2i). Depending on the specimen, the amount of such OHCs varied from a few scattered cells among three rows to one hundred OHCs. The absence of PKC beta II expression in the OHCs generally correlated with an absence of ChAT or synaptophysin (Figure 2h)

labelled efferent terminals. In the most apical region of 4 cochleae out the 14 observed, however, from 12 to one hundred PKC unlabelled OHCs innervated by ChAT (Figure 2 i) or synaptophysin labelled efferent terminals. In the apical OHCs expressing PKC beta II, the length of the postsynaptic site appeared around three fold smaller than that seen in more basal cochlear regions (Compare figures 2j and 2g), corresponding to the smaller size of the efferent synapses in the apex (Pujol et al., 1998).

In the Hensen cells (Figure 2c), PKC beta II appeared organized in cytoplasmic bundles forming a continuous and longitudinal network throughout the outer region of the organ of Corti. Double labelling with rodhamin plalloidin to target the actin, revealed no obvious colocalization of either protein (Figure not shown).

We found immunolabelling for PKC beta II in the lateral cell membrane of the nonsensory Deiters cells (Figure 2 h) and Claudius cells in the cochlear apical turn (1mm long) only.

Expression pattern of PKC beta II in the developing organ of Corti

At PND 5, we observed PKC beta II in the efferent fibres running in the inner spiral bundle though non in the area of the OHCs (Figure 2i) and the Hensen cells. At PND 8 (Figure 2j), PKC beta II appeared at the synaptic pole of the OHCs in the basal cochlear turns. PKC beta II appeared first in the first (internal) row of OHCs then in the second and third (external) turn. In addition, PKC beta II was present in the efferent fibres from the MOC reaching the base of the OHCs throughout the cochlea. By PND 12, the labelling for PKC beta II had declined in the efferent fibres from the MOC under the OHCs while the protein appeared to concentrate at the synaptic pole of all OHCs from the basal and middle turns (not shown). In addition, a faint PKC beta II labelling appeared in the area of the Hensen cells from the basal and middle cochlear regions (not shown). We observed an adult-like pattern of PKC beta II distribution around PND 20 (see above results).

Immunoelectron microscopy

Expression of PKC beta II in the adult cochlea

We observed a diffuse labelling for PKC beta II in efferent endings of the inner spiral bundle, making axodendritic synapses with radial afferent fibres (Figure 3a).

In the OHCs, both DAB (Figure 3b,c) and VIP (Figure 3d) immunolabelling revealed expression of PKC beta II in the postsynaptic membrane facing the large efferent endings from the MOC and in a narrow band (50-100 nm) of adjacent cytoplasm that included the subsynaptic cistern area.

However, due to the sub-optimal preservation of the OHC subsynaptic cisterns after paraformaldehyde fixation and to the chromogen diffusion, it is not possible to establish with certainty the presence PKC beta II in this organelle. We found no labelling facing the afferent dendrites of the type 2 ganglion neurons (Figure 3b, d).

Expression of PKC beta II in the developing organ of Corti

At PND 5 and PND 8 (Figure 3e), in addition to the labelling seen in the efferent terminals of the inner spiral bundle, we found postsynaptic labelling for PKC beta II at the postsynapse facing direct axosomatic contacts between the IHCs and efferent buttons. Here, both the postsynaptic membrane and the subsynaptic cistern appeared to express PKC beta II. At this developmental stage, immunoelectron microscopy failed to detect the presence of PKC beta II in the OHCs (not shown).

The first labelling for PKC beta II was clearly seen at the basal pole of the OHCs from the basal and middle cochlear turns at day 12. At this stage, only a short segment of the postsynaptic membrane facing small efferent endings was labelled (Figure 3f).

The results are summarized in Table 1.

DISCUSSION

This study demonstrates the presence of PKC beta II in different structures of the rat organ of Corti including the efferent nerve terminals of the LOC, the sensory OHCs and the nonsensory epithelial Hensen cells. This pattern of expression suggests diverse roles for this calcium dependant kinase within the organ of Corti. In the Hensen cells, PKC beta II could be involved in a pathway controlling ion homeostasis, whereas in LOC efferent terminals and outer hair cells, it has a likely involvement in the neurotransmission processes on both sides of the synapse. In addition, the presence of PKC beta II within the cochlear efferent supply and the OHCs suggests a potential role in the protection of the cochlea against acoustic trauma, ischemia-induced excitotoxicity and possibly drug ototoxicity.

Presynaptic expression of PKC beta II

Our current results indicate presynaptic expression of PKC beta II in a single population of efferent fibres in the mature cochlea, the LOC efferents in the inner spiral bundle. The LOC efferent neurons modulate the activity of the primary auditory neurons via axodendritic synapses (for review, see Puel et al., 2002). Acetylcholine released by the efferent terminals increases the spontaneous and glutamate-induced firing of the auditory dendrites (Felix & Ehrenberger, 1992; Ruel et al., 2007) while GABA and dopamine inhibit this neural output (Oestreicher et al., 1997; Ruel et al., 2001; 2006). The action of the other LOC neuroactive substances, CGRP, GABA, enkephalins, dynorphins is less well understood (for reviews, see Eybalin, 1993; Puel., 1995; Le Prell et al., 2001).

Presynaptically, PKC is known to mediate **ACh** release at the neuromuscular junction (Santafe et al., 2005) and the control of nicotinic (Malenka et al., 1986), glutamatergic (Brager et al., 2003) and GABAergic (Okada et al., 2004) synaptic transmission in CNS neurons. In these different forms of neurotransmission, PKC may be involved in vesicle trafficking, recruitment, fusion and recycling

through the phosphorylation of various cytoskeletal and membrane substrates (for reviews, see Nichols, 1997; Larsson, 2006). Among its many functions, PKC targets i) the growth associated protein-43 (Gap-43), highly expressed during neuritogenesis and synaptic plasticity, ii) the actin-cross-linking protein MARCKS (Myristolated alanine-rich C-kinase substrate) involved in vesicle transport, iii) the membrane protein dynamin, implicated in membrane retrieval after exocytosis and iv) the membrane protein SNAP-25 (Synaptosome-associated protein-25), necessary for vesicle docking and fusion.

From the present study, it is not possible to establish the presynaptic role of PKC beta II in the LOC efferent endings. This PKC isoform may be involved in the release of one or several neuroactive substances through the phosphorylation of the presynaptic protein 25 (among others: Montiel et al., 2003) present at the plasma membrane of the LOC efferent buttons (Kurc et al., 1998; Bergeron et al., 2005). However, the fact that PKC beta II expression was not restricted at the presynaptic membrane but occurred along the 5-10 μ m terminal segments, suggests that PKC beta II plays an additional or other roles. The LOC efferent innervation remains highly plastic in the adult cochlea, allowing drastic synaptic reorganization following ischemia- or noise-induced excitotoxicity (for review, see Puel et al., 2002). Therefore, PKC beta II could be involved in synaptic plasticity and dendritic outgrowth through the phosphorylation of GAP-43 and/or MARCKS as occurs in CNS neurons (for review, see Larsson, 2006). Interestingly, MARCKS has been detected in the cochlear tissue (Coling et al., 1994) with GAP 43 expression found in LOC efferents, not only during cochlear development but also in the mature organ (Bergeron et al., 2005). Similarly, PKC beta II could be involved in the synaptogenesis of both LOC and MOC efferents during development. In fact, we found PKC beta II expression in the LOC axons as early PND 5, when reorganization of the efferent synapses occurs under the IHCs (Lenoir et al., 1980; Bruce et al., 2000). In the MOC axons, PKC beta II was transiently present around PND 8, i.e. during the formation of the first axosomatic synapses with the OHCs (Lenoir et al., 1980).

Postsynaptic expression of PKC beta II

The prominent finding of this study is the postsynaptic expression of PKC beta II in the OHCs of the mature cochlea. In these OHCs, the anti-PKC beta II antibody specifically labelled the postsynaptic membrane of the MOC efferent axosomatic synapses. This suggests that PKC beta II participates in the efferent control of OHCs. The OHCs form a cochlear amplifier through somatic electromotility powered by the motor protein prestin (for review see Dallos et al., 2006). The MOC feedback regulates the gain of this cochlear amplifier essentially via the release of **ACh** onto the OHCs (for reviews, see Guinan, 2006 and Frolenkov 2006). The **ACh** induces calcium influx via nicotinic alpha9-alpha10 receptors (Elgoyen et al., 1994; 2001) which in turn activate nearby calcium-gated SK potassium channels, resulting in hyperpolarization and inhibition of the hair cells (Blanchet et al., 1996 and for review see Fuchs, 2002).

Postsynaptically, PKC can phosphorylate receptors and regulate voltage-gated channel activity (for review, see Nichols, 1997). In cholinergic systems, PKC mediates the potentiation of nicotinic alpha7 receptor responses in rat hippocampal slices (Ohta et al., 2003) and phosphorylates nicotinic alpha4 receptors transfected in human epithelial cells (Pacheco et al., 2003). In addition, protein kinase CK2 coassembled with small conductance Ca(2+)-activated K⁺ channels, has been shown to regulate channel gating in the rat brain (Bildl et al., 2004). In our study, the precise localization of PKC beta II at the postsynaptic membrane of OHCs, facing the large efferent endings, thus strongly suggests a role in modulating the activity of the ionotropic alpha9-alpha10 receptors and/or the colocalized calcium-gated potassium channels.

Interestingly, our results show that PKC beta II appears at the synaptic pole of the OHCs between PND 8 and PND 12 when the nicotinic response to acetylcholine switches from depolarization (due to inward calcium current) to hyperpolarization (due to strong outward potassium current) in the isolated OHC of the rat cochlea (Dulon & Lenoir, 1996). Such concomitance suggests that PKC beta II plays a role in the functional coupling between receptors and colocalized calcium activated

potassium channels. For instance, the kinase could mediate a calcium-induced calcium release from stores contained in the postsynaptic cistern (Lioudyno et al., 2004). According to the present results, similar forms of PKC beta II postsynaptic activity may occur in developing IHCs which appear to show efferent postsynaptic PKC beta II expression. In fact, the immature IHCs are transiently innervated by MOC cholinergic efferents (Simmons et al., 1996) and cholinergic inputs mediated by alpha9-alpha10 nicotinic receptors and calcium-activated potassium channels inhibit immature IHCs (Simmons & Morley, 1998; Glowatzki & Fuchs, 2000).

Besides Ach, two other substances GABA and CGRP (Le Prell et al., 2001) may play a neurotransmitter or neuromodulator role in the MOC efferents, although their effects on OHCs are still largely unknown. Thus, the possibility that PKC beta II mediates GABA or CGRP neurotransmission in the OHCs cannot be ruled out. Indeed, in the dorsal horn of the spinal cord, the effects of both GABA and CGRP on nociceptive neurons are likely mediated by PKC-second messenger pathways (Sun et al., 2004; Vergnano et al., 2007).

In the apex of the cochlea, a variable proportion of OHCs lacked PKC beta II at the efferent postsynapse, even in the presence of MOC efferent contacts. These findings resemble immature features seen in PND 8 rats when OHCs are labelled for ChAT but not yet for PKC beta II. Pujol et al. (1998) proposed that the apical OHCs undergo an incomplete developmental process. The innervation pattern of apical OHCs shares mainly similarities with that of IHCs essentially innervated by afferent fibres. The present results thus extend to chemical aspects the morphological differences existing between apical OHCs and those located in the more basal cochlear regions. Moreover, our results support the notion that differential regulatory mechanisms exist in the cochlea between the base and the apex (Coling and Schacht, 1991).

Epithelial expression of PKC beta II

PKC beta II showed strong expression throughout the cochlea in the Hensen cells, whereas expression in the Deiters cells and Claudius cells was found only the cochlear apex. Hensen cells, Deiters cells and Claudius cells are nonsensory epithelial cells which participate in the potassium buffering of the OHC micro-environment and serve to cycle potassium back to endolymph (Todt et al., 1999). These functions rely on the K-Cl co-transporter KCC3 (Boettger et al., 2003), and on large gap junction plaques containing connexins 26 and 30 (Zhao & Yu, 2006) that couple the neighbouring nonsensory epithelial cells (for review, see Kikuchi et al., 2000). As PKC plays a role in the regulation of the K-Cl co-transporter (for review, see Adragna et al., 2004) and gap junction permeability (for review, see Solan & Lampe, 2005), it is tempting to speculate that PKC beta II mediates the control of ion transit in Hensen cells and in the apical Deiters and Claudius cells. However, the absence of PKC beta II in Deiters and Claudius cells of the basal and middle cochlear region (i.e. 90% of the whole cochlear partition), also suggests a more specific role of Hensen cells in ion homeostasis.

Conclusion

The present results suggest an involvement of PKC beta II in cochlear efferent neurotransmission and ion homeostasis; both mechanisms that may protect the hair cells and their innervation against injury. PKC beta II could thus provide a target for further experimental protocols aimed at discovering methods to protect the organ of Corti against, or rescue it from, noise injury, drug ototoxicity and ischemia-induced excitotoxicity.

Acknowledgements

We thank Drs. Dominique Joubert and Corinne Quittau for their precious advice related towards this study. Thanks are also due to AngloScribe (Nîmes, France) for correcting the English. Electron microscopy was carried out at the electron microscopy facility at the Centre de Ressource en Imagerie Cellulaire de Montpellier with the assistance of Dr. Chantal Cazevieille, and confocal

imagery at the centre's MRI-IURC facility. The French Ministère de la Recherche et des Nouvelles Technologies provided financial support for cell imagery.

Abbreviations

ACh: acetylcholine

DAG: diacylglycerol

IHC: inner hair cell

GABA: acide gamma-amino-butyrique

CGRP: calcitonin gene related peptide

ChAT: cholineacetyltransferase

LOC: lateral olivocochlear

MOC: medial olivocochlear

MARKS: myristolated alanine-rich C-kinase substrate

OHC: outer hair cell

PBS: phosphate buffer saline

PKC: protein kinase K

SNAP-25: synaptosome-associated protein-25

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LEGENDS

Figure 1. Immunoblot analysis of PND 35 and PND 12 rat cochleae showing two bands at respectively 80 kD, the molecular weight of PKC beta II and 42 kD, the molecular weight of beta actin.

Figure 2. Immunofluorescence for PKC beta II (green), calbindin (blue) and synaptophysin or 200 kD-neurofilaments or ChAT (red) in a mid-modiolar section (**a**) and whole-mount surface preparations (**b-i**) of the organ of Corti. **a:** (PND 35, middle turn) calbindin labels the IHC (I) and the OHCs (O). PKC beta II is expressed in nerve fibres running in the inner spiral bundle (isb), at the basal pole of the OHCs and in the Hensen cells (H). tC: tunnel de Corti; bm: basilar membrane; D: Deiters cells. **b:** (PND 35, middle turn) PKC beta II reactive efferent axons are seen in the ISB, below the calbindin labelled IHCs. PKC beta II and synaptophysin colocalize in most efferent endings. **c:** (PND 55, middle turn) the basal pole of the OHCs contains patches (or disks) of PKC beta II, facing large efferent endings labelled for 200 kD neurofilaments. The Hensen cells are PKC beta II positive. **d:** (PND 25, middle turn) the basal pole of each calbindin positive OHC shows one or several patches of PKC beta II coinciding with synaptophysin labelled MOC efferent endings. In **e** and **f**, confocal images were three-dimensionally reconstructed using a surface rendering technique with the Imaris program. **e:** synaptophysin immunoreactive MOC efferent endings contact the basal pole of two OHCs: the post-synaptic site is positive for PKC beta II. **f:** removal of the red channel reveals the whole surface of the postsynapses within the OHCs. **g:** (PND 35, basal turn). In the OHC basal pole, the expression of PKC beta II is facing the ChAT labelled MOC efferent endings. **h:** (PND 40, apex) only some calbindin positive OHCs are contacted by synaptophysin immunoreactive efferent endings. In these OHCs, a disk of PKC beta II labelling is visible. The insert shows an enlargement of one efferent synapse. OHCs without efferent contacts do not express PKC beta II. Note also the expression of PKC beta II in the lateral cell membrane of Hensen cells (H) and Deiters cells in a deeper focal plane (asterisks). **i:** (PND 35, extreme apex)

Most calbindin immunolabelled OHCs are contacted by ChAT reactive efferent endings but lack PKC beta II expression. **j**: (PND 35, apex) at the synaptic pole of the apical OHCs, the size of the ChAT-PKC beta II immunolabelled efferent synapses are about 3 times smaller than in the basal turn of the cochlea (compare with Figure g). **k**: (PND 5, middle turn) PKC beta II is already expressed in the efferent fibres below the IHCs (upper row of calbindin positive cells) but is not detectable in the area of OHCs, despite the presence of the first synaptophysin immunolabelled efferent endings. **l**: (PND 12, basal cochlear turn) The MOC terminals which contact the calbindin positive OHCs are labelled for synaptophysin. PKC beta II is now present at the base of the OHCs. A few MOC efferent axons express PKC beta II (arrows). Scale bar: a-l = 10 μ m; insert = 1 μ m.

Figure 3. Immunoelectron microscopy for PKC beta II. **a**: (basal turn of a 55 day-old rat cochlea; DAB labelling) in the inner spiral bundle, an immunoreactive LOC efferent ending (e) synaptically contacts a radial afferent dendrite(a) from a type I ganglion neuron. **b**: (basal turn of a 55 day-old rat cochlea; DAB labelling) the basal pole of a OHC (O) is contacted by two MOC efferent terminals (e) making two large axosomatic synapses, and by one afferent dendrite (a) from a type II ganglion neuron. The postsynaptic membrane (the basolateral membrane of the OHC) is labelled for PKC beta II (arrows). No labelling is detectable facing the afferent contact (asterisk). n: OHC nucleus; D: Deiters cell. **c**: high magnification of an axosomatic synapse shown in b. The labelling for PKC beta II is limited to the post-synaptic membrane and to the underlying cytoplasm (arrows). **d**: (middle turn of a 35 day-old rat cochlea; VIP labelling) the basal pole of a OHC (O) is contacted by one MOC efferent ending (e) and by one afferent dendrite from a type II ganglion neuron (a). A punctuate labelling is visible along the postsynaptic membrane and in the cytoplasm facing the efferent ending. Note the absence of labelling facing the afferent contact. **e**: (middle turn of a 8 day-old rat cochlea; DAB) a direct axosomatic synapse between a IHC (I) and an efferent ending (e). The postsynaptic membrane (and possibly the postsynaptic cistern) is labelled (arrowhead). **f**: (middle turn of a 12 day-old rat cochlea; DAB) the basal pole of a OHC (O) is contacted by one

MOC efferent ending (e) and by an afferent terminal from a type II ganglion neuron (a). A small segment of the postsynaptic membrane facing the efferent is labelled for PKC beta II (arrow). Scale bar = 1 μ m.