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Kanamycin alters cytoplasmic and nuclear phosphoinositide signaling in the organ of Corti *in vivo*

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Abstract

Aminoglycoside antibiotics strongly bind to phosphoinositides and affect their membrane distribution and metabolism. Kanamycin treatment also disrupts Rac/Rho signaling pathways to the actin cytoskeleton in the mouse inner ear *in vivo*. Here, we investigate the influence of kanamycin on phosphoinositide signaling in sensory cells (hair cells) of the mouse cochlea. Immunoreactivity to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) decreased in the organ of Corti, especially in outer hair cells, after 3–7 days of drug treatment, whereas imunoreactivity to phosphatidylinositol-4,5-bisphosphate (PIP_2) increased. Immunoreactivity to PIP_2 was present at the apical poles of outer hair cells, but appeared in their nuclei only after drug treatment. Furthermore, nuclear PIP_2 formed a complex with histone H3 and attenuated its acetylation in outer hair cells. In agreement with reduced PIP_3 signaling, phosphorylated Akt decreased in both the cytoplasm and nuclei of outer hair cells after kanamycin treatment. This study suggests that kanamycin disturbs the balance between PIP_2 and PIP_3, modifies gene transcription via histone acetylation and diminishes the PI3K/Akt survival pathway. These actions may contribute to the death of outer hair cells, which is a consequence of chronic kanamycin treatment.

Keywords: Akt, histone, outer hair cells, phosphatidylinositol-4, 5-bisphosphate, phosphatidylinositol-3, 4, 5-trisphosphate, signaling pathways.

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Phosphoinositides in their different incarnations regulate a wide variety of cellular processes at the plasma membrane, in the cytoplasm and in the nucleus (Irvine 2003; Parker 2004; De Matteis *et al*. 2005). Aminoglycoside antibiotics such as neomycin, gentamicin or kanamycin strongly bind to phosphoinositides (Schacht 1979), alter their metabolism (Schacht 1976) and disrupt membrane structures containing polyphosphoinositides (Lodhi *et al*. 1979). These properties have made the drugs useful probes for studying phosphoinositide metabolism and function (Janmey and Stossel 1989; Arbuzova *et al*. 2000; Holz *et al*. 2000). Phosphoinositides may also be cellular targets of aminoglycosides in their adverse effects on tissues, notably in the death of both sensory cells in the inner ear (ototoxicity) and proximal tubule cells in the kidney (nephrotoxicity).

Although early studies of ototoxicity *in vivo* had demonstrated changes in inner ear phosphoinositide metabolism (Orsulakova *et al*. 1976), only recently have more details of potential physiological mechanisms emerged. The phosphatidylinositol-3-kinase/Akt pathway and its downstream effector nuclear factor κB (NF-κB) play a central role in cell growth and survival in many tissues (Song *et al*. 2005; Woodgett 2005) including the inner ear (Nagy *et al*. 2005). NF-κB is suppressed by aminoglycoside-induced insult *in vivo*, and its activation can rescue the sensory hair cells of the cochlea (Jiang *et al*. 2005). Furthermore, aminoglycosides disturb the structural integrity of the actin cytoskeleton in the inner ear via an action on small GTPases (Jiang *et al*. 2006b), another potential link to phosphoinositides because the actin regulatory proteins and the assembly of

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Abbreviations used: DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PBS-T, PBS with 0.1% Tween 20; PI3K, phosphatidylinositol-3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; RIPA buffer, radioimmunoprecipitation buffer.

actin fibers involves these lipids (Cooper and Schafer 2000; Takenawa and Miki 2001).

Phosphoinositides also constitute a nuclear signaling network regulated independently from other cell compartments (Irvine 2003; Martelli *et al*. 2005; Gonzales and Anderson 2006). The precise function of these lipids in the nucleus has yet to be determined but may include complex structural and regulatory roles such as influencing pre-mRNA splicing and chromatin structure (Osborne *et al*. 2001; Boronenkov *et al*. 1998), as well as regulating gene transcription by histone binding (Yu *et al*. 1998). The effect of aminoglycosides on nuclear phosphoinositide signaling *in vivo* has, to the best of our knowledge, not been studied yet.

In this study, we investigate cytoplasmic and nuclear phosphoinositide signaling pathways in the organ of Corti *in vivo* in order to gain a more detailed understanding of aminoglycoside–phosphoinositide interactions. The animal model used is the adult mouse (CBA/J strain), which receives chronic injections of kanamycin that primarily destroy the outer hair cells (Wu *et al*. 2001). The selected dosing regimen yields a slow progressive ototoxic action, which allows the determination of early cellular responses and their relationship to cell death and survival pathways (Jiang *et al*. 2005, 2006a).

Materials and methods

Materials

Kanamycin sulfate was purchased from USB Corporation (Cleveland, OH, USA; Cat. #17924; Lot #110755), ketamine (Ketaset®) from Fort Dodge Animal Health (Fort Dodge, IA, USA), xylazine (TranquiVed®) from Vedco Inc. (St Joseph, MO, USA), and ECL™ for western blotting detection reagents, from GE Health Care (Piscataway, NJ, USA). BenchMark™ Protein ladders were obtained from Invitrogen™ Life Technologies (Carlsbad, CA, USA). Anti-histone H3, anti-acetyl-histone H3 (Lys9), anti-phospho-histone H3 (Ser10), anti-histone H2A, anti-acetyl-histone H2A (Lys5), anti-Akt1/2 and anti-phospho-Akt1/2 (Thr308 and Ser473) polyclonal antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA); anti-PIP_2 and anti-PIP_3 monoclonal IgM were from Echelon Research Laboratories Inc. (Salt Lake City, UT, USA), and anti-PI4Kβ polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Agarose-conjugated protein A/G was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), agarose-conjugated anti-mouse IgM was from Sigma-Aldrich Inc. (St Louis, MO, USA), antibodies to phosphoserine were from Zymed Laboratories Inc. (South San Francisco, CA, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon International (Temecula, CA, USA). Horseradish peroxidase-conjugated secondary antibodies for western blotting were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA), fluorescent secondary antibodies (Alexa 488 and Alexa 546) and rhodamine phalloidin, propidium iodide, as well as Hoechest 33342 were from Molecular Probes Inc. (Eugene, OR, USA). Complete™ mini EDTA-free protease inhibitor cocktail tablets were from Roche Diagnostic GmbH (Mannheim, Germany).

Animals and drug administration

Male CBA/J mice were delivered at an age of 4 weeks from Harlan Sprague–Dawley Co. (Indianapolis, IN, USA) and divided into three groups (saline-treated control and kanamycin treatment for 3 and 7 days). The animals had free access to water and a regular mouse diet (Purina 5025; Purina, St Louis, MO, USA), and were acclimated for 1 week before the start of experimental procedures. Experimental protocols were approved by the University of Michigan Committee on the Use and Care of Animals. Animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine.

Drug dosing followed our earlier protocol for studying aminoglycoside-induced hearing loss (Wu *et al*. 2001). Experimental mice received a dose of 700 mg of kanamycin base/kg body weight twice daily by subcutaneous injection. Cochleae were collected 3 h after the last injection on the third and seventh day. At this time, the drug did not significantly affect auditory function and morphology. Only continuous treatment with the same dose of kanamycin for 11 days or more will result in outer hair cell death (Jiang *et al*. 2006a).

Extraction of total protein

The cochleae were removed rapidly and dissected in ice-cold 10 mM phosphate-buffered saline (PBS). The dissections included the sensory and supporting cells of the organ of Corti, the supporting structures of the lateral wall (stria vascularis and spiral ligament) and the cochlear portion of the spiral ganglion. Tissue from one mouse cochlea was homogenized in ice-cold radioimmunoprecipitation (RIPA) buffer using a micro Tissue Grind Pestle (Kontes Glass Company, Vineland, NJ, USA) for 30 s. The homogenates were kept on ice for 15 min and then centrifuged at 15 000 *g* at 4°C for 10 min.

Extraction of nuclear protein from cochlear homogenates

Cochleae were removed rapidly and dissected in 10 mM ice-cold PBS. Tissues from three mice were pooled and homogenized in cytoplasmic lysis buffer [10 mM sodium HEPES, pH 7.9, additionally containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), 10 mM each of the phosphatase inhibitors NaF and sodium β-glycerophosphate, 1 μg/mL (*p*-amidinophenyl) methanesulfonyluoride, and 1/10 tablet/mL of Complete Mini EDTA-free protease inhibitor cocktail] by using a micro Tissue Grind Pestle for 10 s. The homogenates were kept on ice for 15 min and then centrifuged at 750 *g* at 4°C for 10 min. The crude nuclear pellet was washed twice with a cytosolic lysis buffer and centrifuged each time at 15 000 *g* at 4°C for 5 min. The washed nuclear pellets were resuspended in a nuclear lysis buffer (50 mM Tris-HCl, pH 7.5, containing 10% glycerol, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and the aforementioned phosphatase and protease inhibitors) and kept on ice for 30 min. The suspensions were centrifuged at 15 000 *g* at 4°C for 10 min, and the supernatant was collected as the nuclear protein extract. The nuclear extract was stored at −80°C until analyzed. Protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry

Mice were decapitated and the temporal bones were quickly removed. Cochleae were immediately perfused with and fixed overnight in 4%

paraformaldehyde at 4°C. Cryostat sections of 5 μm were incubated in 0.5% Triton X-100 for 15 min at room temperature (22–24°C) and then washed three times with PBS. The sections were blocked with 10% goat serum for 30 min at room temperature, followed by the application of the primary antibody at 4°C for 72 h. Concentrations of anti-PIP_2, anti-PIP_3, anti-histone H3, anti-acetyl-histone H3 (Lys9), anti-phospho-histone H3 (Ser10), anti-phospho-Akt1/2 (Thr308 and Ser473) and anti-PI4Kβ were 1 : 50, and anti-Akt1/2 was 1 : 100. The sections and surface preparations were washed three times with PBS, and the secondary antibody (either Alexa 488 or Alexa 546 conjugated) at a concentration of 1 : 500 was applied at 4°C overnight in darkness. Finally the preparations were incubated with either Hoechst 33342 or propidium iodide (2 μg/mL in PBS) at room temperature for 40 min for fluorescent visualization of the nucleus. After being washed with PBS, the slides were mounted and photographed with laser confocal microscope (Zeiss LSM 510; Carl Zeiss Microimaging Inc., Thornwood, NY).

Western blot analysis

Either total protein (50 μg each) or nuclear protein (30 μg each) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). A 12% polyacrylamide gel was used for the separation of histone, a 10% gel for Akt1/2, and a 7.5% gel for PI4Kβ. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Pierce, Rockford, IL, USA) and blocked with 5% non-fat dry milk in PBS with 0.1% Tween 20 (PBS-T). The membranes were then incubated overnight with anti-Akt1/2 (1 : 500), anti-phospho-Akt1/2 (Thr308 and Ser473) 1 : 250, anti-histone H3 (1 : 500), anti-acetyl-histone H3 (Lys9) 1 : 500, anti-phospho-histone H3 (Ser10) 1 : 200, anti-histone H2A and anti-acetyl-histone H2A (Lys9) 1 : 250 and anti-PI4-kinase-β (1 : 100) rabbit polyclonal antibodies. After three washes with PBS-T buffer, the membranes were incubated with the secondary antibody (goat anti-rabbit IgG) at a concentration of 1 : 10 000 for 1 h. Following extensive washing of the membrane, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ, USA). The membranes were then stripped and restained with anti-GAPDH at a concentration of 1 : 20 000 to confirm the consistency of protein loads.

Immunoprecipitation

Cochlear nuclear extracts (300 μg) were incubated with 2 μg of anti-PIP_2 antibody in an Eppendorf tube (a minimum of 300 μL per tube). The tube was rotated overnight at 4°C and then 2 μg of agarose-conjugated anti-IgM were added and the tube again rotated overnight at 4°C. Cochlear total proteins (300 μg) were pre-cleared by incubation with 20 μL of protein A/G-agarose beads for 1 h at 4°C. Four μg of anti-PI4Kβ antibody were added to the supernatant, rotated for 6 hr at 4°C, and then 20 μL of protein A/G-sepharose beads were added for incubation overnight at 4°C. Beads were collected by centrifugation for 1 min at 1200 *g* at 4°C and the supernatant was removed. The pellets were washed three times with lysis buffer, and after the final wash the pellets were re-suspended in 20 μL of 2X electrophoresis sample buffer (including 0.5 M Tris-HCl, 4% SDS, 20% glycerol, 0.02% bromophanol blue and 10% β-mercaptoethanol). Subsequently, anti-histone H2A (1 : 250), anti-histone H3 (1 : 500) and anti-phospho-serine (1 : 100) were detected by using western blotting.

Statistical analysis

Data were statistically evaluated by Student's *t*-test and by analyses of variance with a Student's Newman–Keuls test for significance ($p < 0.05$) using PRIMER OF BIOSTATISTICS software (McGraw-Hill Software, New York, NY, USA).

Results

PIP_3 decreases in outer hair cells after kanamycin treatment

In untreated and saline-treated CBA/J mice, PIP_3 was primarily located in the membranes of most cells of the organ of Corti, including outer and inner hair cells, pillar cells and supporting cells. The distribution of staining also suggested its presence in the cytoplasm of supporting cells (Fig. 1a). During kanamycin treatment for 3 days PIP_3 gradually decreased in the membranes of outer hair cells, but there was no obvious change in the inner hair cells, pillar cells and supporting cells (Fig. 1b). After kanamycin treatment for 7 days the cochlear structure was still intact (Fig. 1c), but PIP_3 had disappeared completely from the outer hair cells and the tops of pillar cells (Fig. 1d).

PIP_2 increases in the membranes and nuclei of outer hair cells after kanamycin treatment

The immunostaining for PIP_2 was characterized by a weak punctate presence in the apical portion (cuticular plates) of outer hair cells in saline-treated control mice (Fig. 2). In contrast to the disappearance of PIP_3, PIP_2 increased in the apex of outer hair cells after kanamycin treatment for 3 days forming dense dotted arrays. The staining patterns maintained a similar appearance after 7 days of treatment.

At the nuclear level, PIP_2 was essentially absent (i.e. below the detection threshold) from the nuclei of outer hair cells in control animals (Fig. 3a). As PIP_2 increased in the cells, immunostaining first moved to the edge of the outer hair cell nuclei and then gradually further into the nuclei (Figs 3b and c).

Nuclear PIP_2 forms a complex with and inhibits the acetylation of histone H3

Immunoprecipitation of nuclear extracts from cochlear tissues (including but not limited to the organ of Corti) with an anti-PIP_2 antibody followed by blotting against histone H3 showed that a complex between PIP_2 and histone H3 was formed, increasing in intensity with kanamycin treatment for 7 days (Fig. 4a). However, PIP_2 did not form a complex with either histone H2A or β-actin (data not shown).

Under the same treatment conditions kanamycin decreased the acetylation of histone H3 in the tissue extracts, whereas the total histone did not change (Figs 4b and c). For a

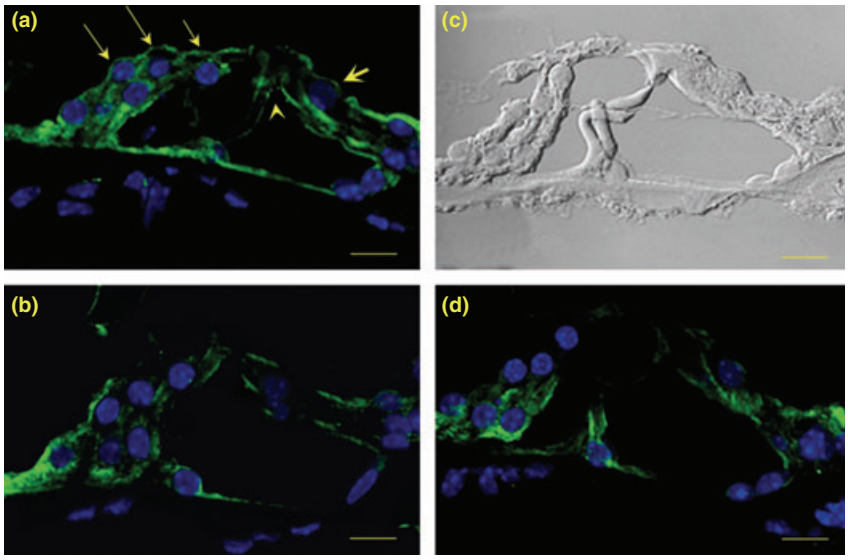

Fig. 1 Kanamycin treatment decreases phosphatidylinositol-3,4,5-trisphosphate (PIP_3) in outer hair cells and pillar cells. PIP_3 was localized immunocytochemically as described in Materials and methods. Immunoreactivity is seen in all cell types of the organ of Corti in the saline-treated control animals (a), including outer hair cells (long arrows), inner hair cells (short arrow), pillar cells (arrowhead) and the surrounding supporting cells. After 3 days of kanamycin treatment, the immunoreactivity of PIP_3 in outer hair cells decreases (b). After 7 days, the structure of the cochlea remained intact (c) but the immunoreactivity of PIP_3 was further attenuated, most notably in outer hair cells and at the top of the pillar cells (d). Green fluorescence indicates PIP_3 immunoreactivity; blue fluorescence shows Hoechst 33342 indicating nuclei. The staining patterns shown are representative of sections from three different animals per condition. Scale bar = 10 μm.

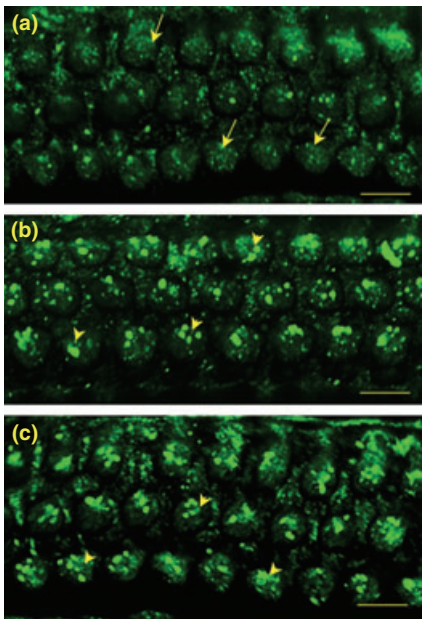

Fig. 2 Phosphatidylinositol-4,5-bisphosphate (PIP_2) increases in the apex of outer hair cells. Surface preparations were stained for PIP_2 immunoreactivity (green) and imaged at the level of the apical plate of outer hair cells. Staining was very weak in saline-treated control mice (a, arrows). After treatment with kanamycin for 3 and 7 days the PIP_2 staining had increased and formed dots (b and c, arrowheads). This figure is representative of three individual animals for each condition. Scale bar = 10 μm.

localization of acetylated histone H3 we then stained a surface preparation of the organ of Corti for immunoreactivity to acetyl-histone H3 (Fig. 5). Staining was heavy in the nuclei of outer hair cells of control animals and decreased during kanamycin treatment for 3 days, further diminishing at 7 days. In contrast, levels of phospho-histone H3 did not change after kanamycin treatment for 3 and 7 days (data not shown).

Kanamycin treatment inhibits phosphorylation of Akt in outer hair cells

The phosphorylation of Akt reflects the activity of the phosphatidylinositol-3-kinase pathway. As PIP_3 decreased after kanamycin treatment, we examined the total Akt1/2 and phosphorylated Akt1/2 (Thr308 and Ser473) in inner ear tissue extracts (Fig. 6). Kanamycin did not affect the level of total Akt1/2. However, phosphorylated Akt1/2 was reduced by kanamycin treatment for 3 days and reduced further after 7 days of treatment.

In sections of the cochleae of saline-treated control animals, phosphorylated Akt1/2 was localized in both the cytoplasm and nuclei of outer and inner hair cells, and supporting cells (Fig. 7a). After kanamycin treatment for 3 days, phosphorylated Akt1/2 decreased gradually in both the cytoplasm and nuclei of outer hair cells (Fig. 7b), and decreased even further after treatment for 7 days. Although phosphorylated Akt1/2 essentially disappeared from the

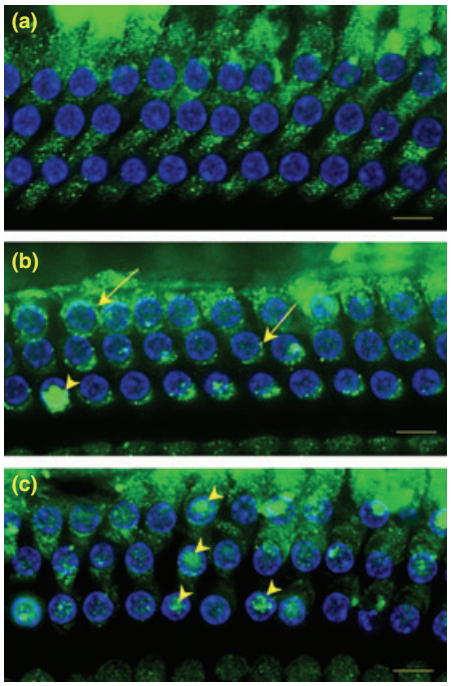

Fig. 3 Kanamycin increases phosphatidylinositol-4,5-bisphosphate (PIP_2) in the nuclei of outer hair cells. Surface preparations (as in Fig. 2) were imaged at the nuclear level (blue, nuclear staining with Hoechst 33342). Nuclei of outer hair cells were negative for PIP_2 in saline-treated control mice (a). After kanamycin treatment for 3 days, the PIP_2 immunoreactivity appeared in the nuclear membranes (b, arrow), and only a few cells showed a strong signal for PIP_2 immmunoreactivity inside the nuclei (b, arrowhead). After kanamycin treatment for 7 days most nuclei of outer hair cells displayed visible PIP_2 staining (c, arrowhead). Panels are representative of sections from three different animals per condition. Scale bar = 10 μm.

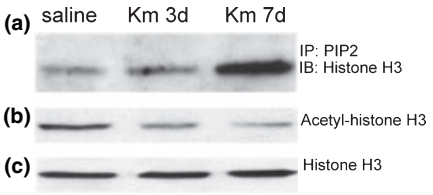

Fig. 4 Kanamycin treatment increases the formation of a complex between phosphatidylinositol-4,5-bisphosphate (PIP_2) and histone H3, and inhibits histone H3 acetylation. Nuclear extracts from cochlear homogenates were prepared for western blotting and immunoprecipitation as described in Materials and methods. Immunoprecipitated bands indicating a complex between PIP2 and histone H3 were slightly increased by kanamycin treatment for 3 days, and significantly so by treatment for 7 days (a). Levels of acetyl-histone H3 on western blots were gradually reduced by kanamycin treatment (b), whereas total histone H3 did not change (c). Saline indicates saline-treated control treatment; Km 3d and 7d indicate kanamycin treatment for 3 and 7 days, respectively. One representative experiment of three is shown.

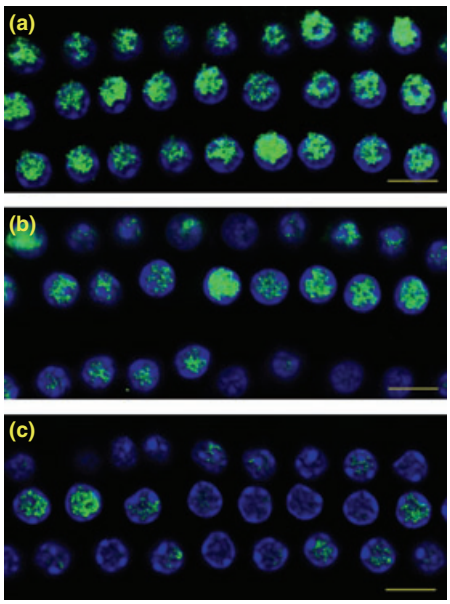

Fig. 5 Immunoreactivity of acetyl-histone H3 decreases in the nuclei of outer hair cells after kanamycin treatment. Surface preparations were stained with acetyl-histone H3 (green) as described in Materials and methods. Immmunoreactivity of acetyl-histone H3 was expressed in all nuclei of outer hair cells in saline-treated control mice (a). After kanamycin treatment for 3 days, immunoreactivity of acetyl-histone H3 was decreased, and disappeared from some cells (b). After kanamycin treatment for 7 days, most of the outer hair cell nuclei displayed no immunoreactivity (c). Blots shown are representative of three different animals per condition. Scale bar = 10 μm.

outer hair cells, it remained present in supporting cells after 7 days treatment of kanamycin (Fig. 7c).

Discussion

The present study clearly indicates phosphoinositides as *in vivo* targets of aminoglycosides in the cochlea. Of particular interest is the drug-induced shift between the levels of PIP_2 and PIP_3, and the emergence of PIP_2 in the nuclei of outer hair cells. Both of these events appear to have distinct consequences for the reaction of the cell to a drug challenge.

PIP_3 is a key regulator of several pathways, including Akt as a prominent downstream target. This pathway may serve the maintenance of homeostasis in the survival of cells either during development or under stress, and it may serve such a function in the mammalian cochlea (Jiang *et al*. 2005; Nagy *et al*. 2005). As the level of PIP_3 is decreased by kanamycin treatment in the cochlea, the phosphorylation of Akt correspondingly is attenuated to the point that it escapes detection in immunocytochemical staining of outer hair cells. This predominant attenuation of the pathway in outer hair cells is significant in the context that these cells are the

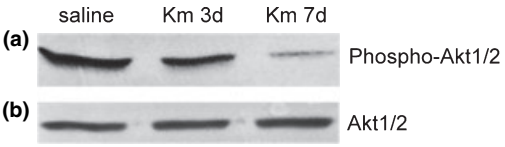

Fig. 6 Kanamycin inhibits the activation of Akt in the cochlea. Phospho-Akt1/2 (Thr308 and Ser473) in whole cochlear extracts was detected by western blotting. The level of phosphorylated Akt1/2 was reduced by kanamycin treatment (a). In contrast, the levels of total Akt1/2 did not change (b). Blots are representative of three individual animals for each condition.

primary targets of the ototoxic actions of aminoglycosides (Forge and Schacht 2000), and will die during continued drug treatment (Wu *et al*. 2001; Jiang *et al*. 2006a). Surviving supporting cells, in contrast, have increased levels of phospho-Akt in their nuclei. Although the nuclear presence of the PI3K/Akt pathways has been documented, little is known about its functional significance (Neri *et al*. 2002). Some targets of Akt, notably the forkhead family of transcription factors, reside in the nucleus where they promote the transcription of apoptotic genes (Nicholson and Anderson 2002). Upon phosphorylation by active phospho-Akt, these factors may be exported and sequestered in the cytoplasm. It is intriguing to speculate that nuclear phospho-Akt therefore contributes to the survival of supporting cells during a drug challenge.

Although the nuclear role of phosphoinositides is less firmly established than their plasma membrane/cytoplasmic pathway, the lipids may contribute to the regulation of gene expression by modulating DNA replication, gene transcription and apoptosis (Irvine 2003; Martelli *et al*. 2005; Gonzales and Anderson 2006). Histones are potential targets of nuclear phosphoinositides as PIP_2, but not PIP_3, binds to histone H1 and histone H3, regulating gene transcription (Yu *et al*. 1998). Histone binding increases as nuclear PIP_2 increases with kanamycin treatment, correlating with an attenuation of histone acetylation. Histone modification is an important switch in the control of gene expression (Verdone *et al*. 2005). Furthermore, phosphoinositides can regulate histone phosphorylation and this phosphorylation can result in cell apoptosis (Enomoto *et al*. 2003; Prigent and Dimitrov 2003). The unchanged levels of phospho-histone H3 after kanamycin treatment for 3 and 7 days is in agreement with our previous finding that cell death in the inner ear does not occur until after either 10 or 11 days (Jiang *et al*. 2006a). Histone acetylation also regulates gene expression but, as far as we can determine, phosphoinositides have not yet been linked to histone acetylation. It is interesting, however, that oxidative stress can inhibit histone acetylation (Berthiaume *et al*. 2006) because oxidative stress is one of the mechanisms by which aminoglycosides exert their ototoxic actions (Priuska and Schacht 1995; Lesniak *et al*. 2005).

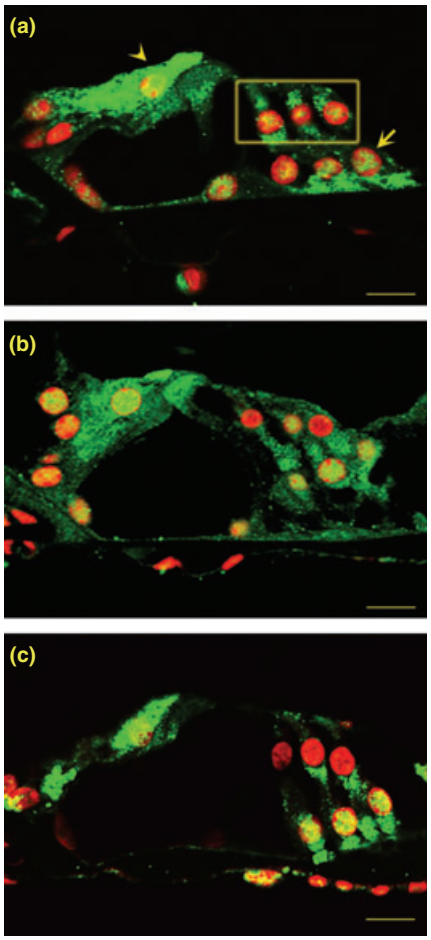

Fig. 7 Kanamycin treatment decreases phosphorylated Akt in outer hair cells and pillar cells. Immunoreactivity of phosphorylated Akt1/2 was localized to the cytoplasm and nuclei of outer hair cells (a, box), supporting cells (a, arrow), inner hair cells (a, arrowhead) and pillar cells of control animals (a). After kanamycin treatment for 3 days, phosphorylated Akt1/2 decreased primarily in both the cytoplasm and nuclei of outer hair cells (b). After kanamycin treatment for 7 days, the immunoreactivity of phosphorylated Akt1/2 was very weak in the cytosol of the outer hair cells and pillar cells and had disappeared from the nuclei of outer hair cells, but increased in the nuclei of Deiters cells (c). The green staining highlights phospho-Akt1/2 (Thr308 and Ser473); the red staining highlights propidium iodide for nuclei. Sections are representative of three different animals examined per condition. Scale bar = 10 μm.

Our results also add to our knowledge of phosphoinositides in the inner and outer hair cells in particular. Drug effects on these lipids in the cochlea have long been recognized (Orsulakova *et al*. 1976), as has their presence in outer hair cells (Williams *et al*. 1987; Montcouquiol and Corwin 2001; Hirono *et al*. 2004). Their intracellular localization, however, has been disputed, possibly because of the different detection methods employed. PIP_2 is often detected by transfecting cells with plasmids that direct expression of green fluorescent

protein (GFP)-tagged pleckstrin homology (PH) domains (Varnai and Balla 1998), a procedure which may have some limitations (Balla *et al*. 2000). In contrast, anti-PIP_2 antibodies can efficiently and selectively recognize PIP_2 (Fukami *et al*. 1988; Thomas *et al*. 1999), including PIP_2 present in the hair bundles of sensory cells (Hirono *et al*. 2004). Our localization of PIP_2 in the membranes and the apical plates of outer hair cells is in agreement with the literature (Hirono *et al*. 2004) but, in addition, we show the dynamic behavior of phosphoinositides and their nuclear presence.

We cannot say with certainty which reactions are responsible for the shift in PIP_2 and PIP_3 in the hair cells and the nuclear appearance of PIP_2 during drug treatment. *In vitro*, aminoglycosides interfere with polyphosphoinositide metabolism by binding to the polar head groups of these lipids (Schacht 1976; Lodhi *et al*. 1979), but their actions *in vivo* will depend, in part, on tissue penetration and distribution of the drugs vs. the compartmentalization of phosphoinositides. The most parsimonious explanation, nevertheless, is an inhibition of the kinase reaction(s) from PIP_2 to PIP_3 leading to an accumulation of PIP_2 and a corresponding decrease in PIP_3 in the membranes. For the nuclear emergence of PIP_2, a similar mechanism may be assumed as aminoglycosides may enter the nucleus (Sha and Schacht, unpublished). Furthermore, as all enzymatic machinery and potential precursors are present in the nucleus, a translocation of increased PIP_2 from other cell compartments seems unlikely. Interestingly, phosphatidylinositol-4-kinase, which synthesizes PIP_2 from phosphatidylinositol-5-phosphate, was not found immuno-cytochemically in the nuclei of outer hair cells in both normal and kanamycin-treated mice (data not shown).

In summary, this study suggests that kanamycin alters phosphoinositide signaling by affecting PIP_2 and PIP_3. Increased nuclear PIP_2 increases complex formation between PIP_2 and histone H3, attenuating the acetylation of histone H3, which may induce an inhibition of gene transcription. A consequence of low levels of PIP_3 may be the inhibition of the activities of the PIP_3/Akt pathway, contributing to outer hair cell death.

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