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RESEARCH PAPER

Glucocorticoid-stimulated, transcription-independent release of annexin A1 by cochlear Hensen cells

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Background and purpose: The current clinical strategy to protect the auditory organ against inflammatory damage by migrating leukocytes is the local delivery of glucocorticoids. However, the mechanism by which glucocorticoids confer this protection remains unknown. Therefore, we investigated the cellular and molecular targets of glucocorticoids in the cochlea that could be involved in preventing leukocyte migration.
Experimental approach: We used microscopy as well as immunocytochemical and microfluidic techniques to elucidate the effect of dexamethasone, hydrocortisone and prednisolone on the cellular and intracellular distribution of annexin A1 (ANXA1) – a glucocorticoid target known to inhibit leukocyte migration by receptor-mediated signalling – in the cochlea and isolated cochlear cells of guinea pigs.
Key results: All the cells lining the scala media – the cochlear compartment containing the auditory organ – express ANXA1 and the ANXA1 receptor FPR2/ALX is present in the scala media, as well as in other cochlear ducts. The majority of ANXA1 in the scala media is stored inside lipid droplets within cochlear Hensen cells. Glucocorticoids activate a myosin IIC-mediated mechanism that drives ANXA1 from the lipid droplets to the apical region of the Hensen cells, where ANXA1 is released to the external milieu by a process involving ABC transporters.
Conclusions and implications: These findings suggest that ANXA1 could be a major mediator of the anti-inflammatory effects of glucocorticoids in the cochlea and identify new molecular targets for prevention of sudden sensorineural hearing loss.
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Keywords: glucocorticoids; annexin A1; myosin IIC; lipid droplets; ABC transporters; Hensen cells; cochlea; inflammation; guinea pigs

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ANXA1, annexin A1; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; OC, organ of Corti; SM, scala media; ST, scala tympani; SV, scala vestibuli

Introduction

The mammalian cochlea is a coiled structure divided into three parallel ducts, the scala vestibuli (SV), the scala tympani (ST) and the scala media (SM) (Figure 1A). The SM, which is delineated by Reissner's membrane above and the basilar membrane below, contains the organ of Corti (OC), the auditory organ composed of sensory cells (inner and outer hair cells) as well as Hensen, Pillar and Deiter cells, all with presumed supporting roles. The stria vascularis covers the lateral wall of the SM, whereas the spiral ligament anchors the stria vascularis and connects the basilar membrane to the lateral wall. Tight junctions between the cells making up the structures surrounding the SM prevent potassium-rich endolymph filling in this duct from leaking into the sodium-rich perilymph of the SV and ST. The ionic gradient between endolymph and perilymph helps generate an electric potential (~80 mV), called the endocochlear potential, which is essential for auditory function.

Leukocyte migration to sites of injury or infection is a defining step of inflammatory responses. In the mammalian cochlea, however, leukocyte migration into the auditory

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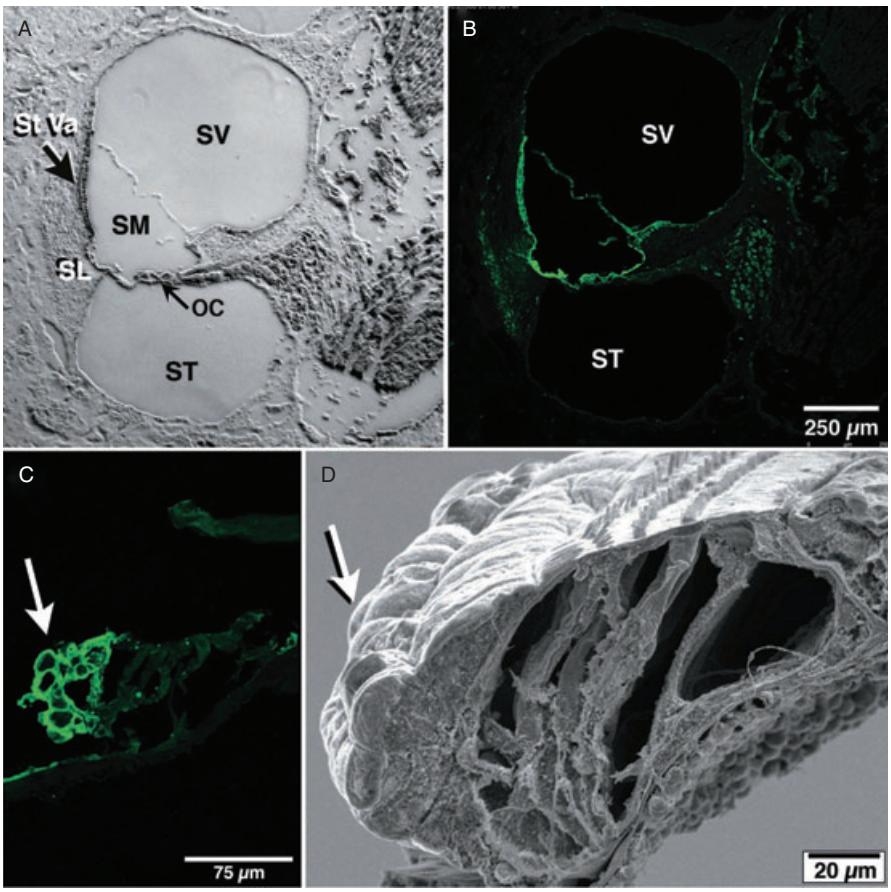

Figure 1 *ANXA1 is expressed in guinea pig cochleae.* (A) Frozen section of a guinea pig cochlea. (B) Immunofluorescence of the same section shown in (A) labelled with anti-ANXA1 (green). (C) ANXA1 is abundantly expressed by Hensen cells (arrow), but not by other cells of the organ of Corti (OC). (D) SEM micrography showing localization of Hensen cells (arrow) in the 3-d structure of the OC (compare with C). ANXA1, annexin A1; SL, spiral ligament; SM, scala media; ST, scala tympani; St Va, stria vascularis; SV, scala vestibuli.

organ must be prevented because it may abolish the endocochlear potential by disrupting the tight-junction barrier at the OC luminal border (anatomically defined as the reticular lamina). Loss of the endocochlear potential leads to apoptosis of sensorimotor outer hair cells and irreversible, profound deafness. Inflammatory responses usually start at the lateral wall with leukocytes migrating from the spiral ligament into the SV and ST but never penetrating into the SM (Hirose *et al.*, 2005; Tornabene *et al.*, 2006). The mechanism used by the cochlea to prevent migration of polymorphonuclear leukocytes and macrophages and complete the resolution phase of the inflammatory response is still unknown, except by the clinically exploited fact that it can be stimulated by glucocorticoids.

Annexin A1 (ANXA1), a lipid and Ca^{2+} binding protein, has been implicated in many aspects of the innate and adaptive immune system (D'Acquisto *et al.*, 2008; Perretti and D'Acquisto, 2009) and is also a well-known molecular target of glucocorticoids (Flower and Blackwell, 1979; Blackwell *et al.*, 1980; Perretti and D'Acquisto, 2009). Specifically, ANXA1 is known to inhibit leukocyte migration and extravasation by receptor-mediated signalling as well as to regulate cell death signalling and the phagocytic clearance of apoptotic cells (Perretti *et al.*, 1996; Perretti and Gavins, 2003; Parente and Solito, 2004; D'Acquisto *et al.*, 2008). ANXA1 is highly expressed in a few differentiated cell types, such as macrophages, monocytes, folliculostellate pituitary cells and glia (John *et al.*, 2004; Kamal *et al.*, 2005; Omer *et al.*, 2006), and is apparently absent in many other cells (Fava *et al.*, 1989). Studies using cell fractionation and immunogold labelling indicate that ANXA1 exists in three distinct pools inside the cells: (i) in the cytoplasm; (ii) embedded in membrane structures; and (iii) attached to the outer surface of the plasma membrane (Peers *et al.*, 1993). Despite lacking a cleavable hydrophobic signal sequence in its amino terminus, in some cell types externalization of ANXA1 is stimulated by glucocorticoid treatment in a time- and concentration-dependent manner (Solito *et al.*, 2003) that does not involves the classical secretory pathway (Wein *et al.*, 2004; Omer *et al.*, 2006). Extracellular ANXA1 binds to a G protein-coupled receptor termed FPR2/ALX (Ye *et al.*, 2009), a protein belonging to a class of receptors with functions specific to the innate immune system (Chiang *et al.*, 2006; Panaro *et al.*, 2006). Thus, the presence in the OC of a cell type expressing ANXA1, and with the ability to release it when stimulated by glucocorticoids, could provide important clues about the mechanism that regulates inflammatory responses in the mammalian cochlea.

Guinea pig Hensen cells are high columnar cells characterized by abundant, prominent lipid droplets in the cytoplasm, which form the outer edge of the OC (Figure 1C,D

and Figure S1). Hensen cells have been associated with potassium recycling in the cochlea (Wangemann, 2002), and some researchers speculated that they might play a role in protecting sensory hair cells from noise trauma (Merchan *et al.*, 1980; del Canizo-Alvarez *et al.*, 1987; Flock *et al.*, 1999). It has been reported that exposure to high-intensity noise induces release of lipid droplets from guinea pig Hensen cells (Merchan *et al.* 1980; del Canizo-Alvarez *et al.*, 1987), and produces a significant reduction in the height of these cells in a murine model (Wang *et al.*, 2002). It has also been reported that Hensen cells of neonatal mice express MYH14 (Donaudy *et al.*, 2004), the gene encoding non-muscle myosin IIC, a motor protein with still obscure functional roles (Clark *et al.*, 2007; Wylie and Chantler, 2008). The actual functional and structural roles of Hensen cells, however, still remain to be understood.

In order to understand how the inner ear copes with common inflammatory responses, we need to identify the glucocorticoid-activated mechanism that inhibits the migration of leukocytes into the endolymph-filled SM, and what cells in the OC are involved in this response. In this report we present evidence that ANXA1 is expressed by epithelial cells enclosing the guinea pig SM; that the ANXA1 receptor FPR2/ALX is widely distributed in cochlear epithelia; that cochlear Hensen cells store significant amounts of ANXA1 inside cytoplasmic lipid droplets; and that glucocorticoids activate a cellular mechanism driven by myosin IIC that results in the release of ANXA1 from cochlear Hensen cells via ABC transporters. Based on these experimental results, we propose that the anti-inflammatory effects of glucocorticoids in the cochlea are mediated by ANXA1, which could play a major role in preventing leukocytes from invading the OC and facilitating the resolution of inflammatory responses in the mammalian inner ear.

Methods

Cochlear preparation and Hensen cell isolation

All animal care and experimental procedures were approved by the House Ear Institute's Institutional Animal Care and Use Committee. Cochleae were obtained from young guinea pigs (*Cavia porcellus*, 200–300 g) killed with CO_2. Temporal bones were placed in Leibowitz L-15 (Invitrogen, Chicago, IL, USA) in a Petri dish and the bulla widely opened. The cochlear spiral was removed and placed in a Petri dish to be either exposed to pharmacological agents (see below) for different periods and then fixed for confocal or electron microscopy observation, or micro-dissected to isolate Hensen cells (Figure S1). *In situ* and isolated Hensen cells were exposed to dexamethasone (1, 10 and 100 nM), hydrocortisone (1 and 100 nM), prednisolone (1 and 100 nM), blebbistatin (100 μM), monensin (10 μM), nocodazole (3.5 μM), glyburide (100 μM) – all from Sigma (St. Louis, MO, USA) – or brefeldin A (1.5 μM. Invitrogen), for different periods as described in the text.

Frozen sections of guinea pig OC

Otic bullae were fixed with 4% paraformaldehyde overnight at 4°C, then washed out with 10 mM phosphate buffer saline (PBS) for 30 min, and decalcified with 120 mM EDTA for 4 weeks. Decalcified cochleae were washed with PBS twice for 30 min, placed first in 15% sucrose solution for 20 min and then moved to a 30% sucrose solution at 4°C overnight. The cochleae were molded with OCT embedding medium (Sakura Finetek USA, Torrance, CA, USA) in proper orientation, frozen in liquid nitrogen and sectioned in a cryostat at 10 μm thickness. The sections were stored at –20°C until used. Sections were labelled with anti-ANXA1 (Invitrogen) at 1:100 dilution following standard protocols, and observed with a TCS-SP5 Broadband Spectra laser confocal microscope with 10× and 20× objectives (Leica Microsystems Inc., Deerfield, IL, USA).

Confocal microscopy

Excised cochlear spirals and isolated Hensen cells, after being exposed for variable periods either to L-15 alone (Control) or L-15 plus dexamethasone, hydrocortisone, prednisolone, blebbistatin, monensin, nocodazole, glyburide or brefeldin A (alone or combined), were fixed in 4% paraformaldehyde (EMS, Fort Washington, PA, USA) in PBS for 2 h, and processed for confocal microscopy following standard procedures. Anti-ANXA1 anti-rabbit (Invitrogen) and anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-myosin IIA (Sigma), anti-myosin IIB (Abcam, Cambridge, MA, USA), anti-myosin IIC (kindly provided by Dr. Robert Adelstein, NIH) and anti-ABCA1 (Novus Biologicals, Littleton, CO, USA) were used as primary antibodies at 1:100 dilution. Rhodamine phalloidin from Molecular Probes-Invitrogen (Eugene, OR, USA), Nile Red and DAPI from Sigma were used to stain actin, lipids and cell nuclei, respectively, and Alexa 488 (anti-goat and anti-rabbit) and Alexa 546 (anti rabbit) from Molecular Probes–Invitrogen were used as secondary antibodies at 1:500/1:1,000 dilutions. Samples were observed with a TCS-SP5 Broadband Spectra laser confocal microscope with a 63 × (NA = 1.2) objective (Leica Microsystems, Deerfield, IL, USA). Images were cropped, resized, and brightness and contrast over the whole image adjusted where necessary, using Photoshop (Adobe Software).

Transmission electron microscopy

Isolated Hensen cells were chemically fixed by immersion in buffered 2.5% glutaraldehyde, and then subjected to a rapid microwave-assisted dehydration and embedding protocol (Webster, 2007). The processing was carried out using a BioWave processor (Ted Pella Inc., Redland, CA, USA) and consisted of short exposures to osmium tetroxide, acetone and unpolymerized epoxy resin in the presence of microwaves. The cells were embedded and polymerized in Eponate 12 using overnight heat polymerization at 60°C. Embedded cells were thin sectioned, mounted on metal specimen grids, stained with uranyl acetate and lead citrate, and examined with a CM 120 BioTwin TEM (FEI Inc., Hillsboro, OR, USA) operating at 80 kV. For immunocytochemistry, cells were chemically cross-linked with either 4% formaldehyde (w/v using paraformaldehyde powder) in 200 mM HEPES buffer (pH 7.4) or with 4% buffered formaldehyde containing 0.1% glutaraldehyde in the HEPES buffer. The specimens were embedded in Lowicryl HM20 resin using a Progressive

Lowering of Temperature protocol in a freeze substitution device (AFS2, Leica Microsystems, Deerfield, IL, USA). For this, specimens were immersed in increasing concentrations of ethanol and simultaneously cooled until they reached a temperature of –50°C. The specimens were soaked in increasing concentrations of resin in alcohol with final incubations in 100% resin at –50°C. Finally, the specimens were placed in fresh resin and then polymerized at –50°C by exposure to ultraviolet light. Sections were prepared with an ultramicrotome (UC6, Leica Microsystems, IL, USA) and collected onto metal specimen grids. All sections prepared for immunolabelling were exposed to immuno-reagents (primary antibody followed by colloidal gold probe) using iterative methods by floating the grids, section-side down on small drops of reagent (Webster and Webster, 2007). Epoxy resins or Lowicryl-embedded sections were labelled with goat anti-ANXA1 (Santa Cruz), followed by rabbit anti-goat bridging antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and protein A gold (PAG, University of Utrecht, Utrecht, the Netherlands). Other sections were labelled with rabbit anti-ANXA1 antibodies (Invitrogen) and PAG. All immunolabelled sections were examined using a transmission electron microscope operating at 80 kV (BioTwin CM120, FEI-Philips, Hillsboro, OR, USA). Images were cropped, resized, and brightness and contrast over the whole image adjusted where necessary, using Photoshop (Adobe Software).

Scanning electron microscopy

Whole cochleae were chemically fixed in 2.5% buffered glutaraldehyde and processed using the TOTO method (Jongebloed *et al.*, 1999), an iterative exposure to tannic acid, osmium tetroxide, sodium thiocarbohydrazide and osmium tetroxide. This was followed by dehydration in ethanol, critical point drying, mounting on a specimen stub and, if required, coating with a 6 nm thick film of platinum. Specimens were examined in a scanning EM (XL-30 SFEG, FEI Inc., Hillsboro, OR, USA) operating at 5 kVa. Images were cropped, resized, and brightness and contrast over the whole image adjusted where necessary, using Photoshop (Adobe Software).

Protein analysis

To quantify the amount of ANXA1 released by Hensen cells, isolated cells were stabilized in PBS for 2 h and then exposed to glucocorticoids. Samples (150 μL) of cell medium were collected at 0 (before adding glucocorticoids), 5, 10, 15, 20, 25 and 30 min of exposure. ANXA1 present in the samples was immunoprecipitated with Dynabeads™ Protein A, separated using a DynaMag-2 magnetic separator (Invitrogen) following manufacturer's published protocols, and quantified with microfluidic techniques (see below). In similar experiments, Hensen cells were exposed to blebbistatin for 30 min before addition of glucocorticoids and for the total length of the experiment. To quantify the amount of ANXA1 in Hensen cell cytoplasm, isolated cells – after being exposed for variable periods either to PBS alone (control) or glucocorticoids and blebbistatin (alone or combined) – were lysed and homogenized at 4°C in a 50 mM Tris buffer solution, pH 7.4, containing Triton X-100, 0.5%; CHAPS, 4%; EDTA, 2 mM; NaCl, 100 mM; vanadate, 1 mM; 10 $\mu L \cdot mL^{-1}$ of 0.1M PMSF; 2 $\mu L \cdot mL^{-1}$ of 10 $mg \cdot mL^{-1}$ leupeptin and 2 $\mu L \cdot mL^{-1}$ of 10 $mg \cdot mL^{-1}$ aprotinin. The total protein content of the samples was measured using the BCA method (Thermo Scientific, Inc., Rockford, IL, USA) and a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). ANXA1 was immunoprecipitated from samples with identical total protein amounts as described above.

Microfluidic studies. Immunoprecipitated ANXA1 samples (5 μL) were charged (by duplicate) in 96-well plates, and digitally quantified using the HT Protein Express LabChip™ kit in a LabChip™ 90 System (Caliper Life Science, Hopkinton, MA, USA). In every study, data from three independent experiments were analysed with the DataViewer Software (Caliper LS).

ELISA assay

The quantitative analysis of the levels of lipoxin A_4 (LXA_4) released by both cochlear spirals and isolated Hensen cells was performed using the LXA_4 ELISA Kit (Neogen Corp., Lexington, KY, USA) following manufacturer's protocols. Briefly, cochlear spirals and isolated Hensen cells were exposed to glucocorticoids for different periods (0, 5, 10, 15 and 30 min), removed from the media, and the liquid collected and added to the provided 96-well microplate in duplicate. Next, the diluted enzyme conjugate (provided) was added to the wells and the mixture incubated at room temperature for 1 h. During the incubation, competition for binding sites between the enzyme conjugate and LXA_4 in the sample is taking place. The bound enzyme conjugate was detected by the addition of substrate, which generates an optimal color after 30 min. Quantitative results were obtained by measuring and comparing absorbance readings at 650 nm of the wells with the samples and those with the standards, using a SpectraMax M5 microplate reader (Molecular Devices).

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Isolated Hensen cells were lysed at 4°C and proteins separated by one-dimensional SDS-PAGE. Protein bands were stained with Bio-Safe Coomassie blue (Bio-Rad Laboratories), excised, digested with sequencing grade trypsin (Promega Corporation, Madison, WI, USA) and analysed by nano-LC-ESI-MS/MS using a LCQ Deca XP ProteomX System (Thermo Electron Corp., San Jose, CA, USA). All MS/MS spectra were searched with SEQUEST™ algorithm-based Bioworks 3.3 (Thermo Finnigan) against a database created by extracting mouse protein sequence entries from NCBI ftp site as previously described (Chakravarti *et al.*, 2008).

Statistical analysis

One-way and two-way ANOVA techniques were used to analyse the data. $P < 0.05$ was selected as the criterion for statistical significance.

Results

ANXA1 is expressed by cells enclosing the guinea pig SM

Initial results in an ongoing project aimed at generating a proteome database of guinea pig OC cells using LC-ESI-MS/MS indicated that Hensen cells express ANXA1 (Table S1). Therefore, we used immunolabelling and confocal microscopy to investigate the expression of ANXA1 and its receptor (FPR2/ALX) in the whole guinea pig cochlea. We found ANXA1 labelling associated with cells making up all the structures enclosing the guinea pig SM, a localization that could be key in preventing leukocyte migration into the OC, and conspicuously absent in ST and SV (Figure 1B). ANXA1 receptors, in contrast, were found in the SM as well in the cells lining the ST and the SV (Figure 2A). In the OC, anti-ANXA1 only labelled Hensen cells. The other cellular components of the OC – the sensory inner and outer hair cells, Deiters and Pillar cells – did not label with anti-ANXA1 antibodies (Figure 1C,D). These cells, however, expressed the ANXA1 receptor FPR2/ALX (Figure 2B–D). We speculate that the expression of FPR2/ALX in these OC cells facilitates their response to ANXA1-mediated signals.

Glucocorticoids induce release of ANXA1 from Hensen cells

We investigated the effect of three glucocorticoids – dexamethasone, (the most commonly used glucocorticoid to treat sudden sensorineural hearing loss), hydrocortisone and prednisolone – on guinea pig cochleae. We found that in all three instances, 5 min exposure to glucocorticoids (10 and 100 nM) resulted in an increase of anti-ANXA1 labelling in the cytoplasm of Hensen cells as well as labelling of the apical surface of outer hair cells and Pillar cells, suggesting binding of released ANXA1 to their receptors (Figure 3A,B). After prolonged exposure to glucocorticoids, Hensen cells appeared to be depleted of ANXA1 and labelling was mostly associated with the apical surface of the OC cells that express the ANXA1-receptor (Figure 3C). In isolated Hensen cells, we found that 3 min exposure to glucocorticoids results in a loss of anti-ANXA1 labelling in the cytoplasm. Five min after exposure to glucocorticoids, ANXA1 was again detected in the Hensen cell cytoplasm, especially near lipid droplets. After 10 min, labelling was observed near the apical surface, and in 20 min most of the Hensen cells were again devoid of ANXA1 labelling (Figure 3D–F). No recovery was evident after 30 min exposure to glucocorticoids.

Note that Figure 3A shows a sample triple-labelled with anti-ANXA1 (green), DAPI (blue) and rhodamine-phalloidin (red), and lipid droplets are observed as 'holes'. However, not every 'hole' in this image corresponded to lipid droplets. To illustrate this fact, and the effect of glucocorticoids on the number and size of lipid droplets inside Hensen cells, samples in Figure 3B,C were triple-labelled with anti-ANXA1 (green),

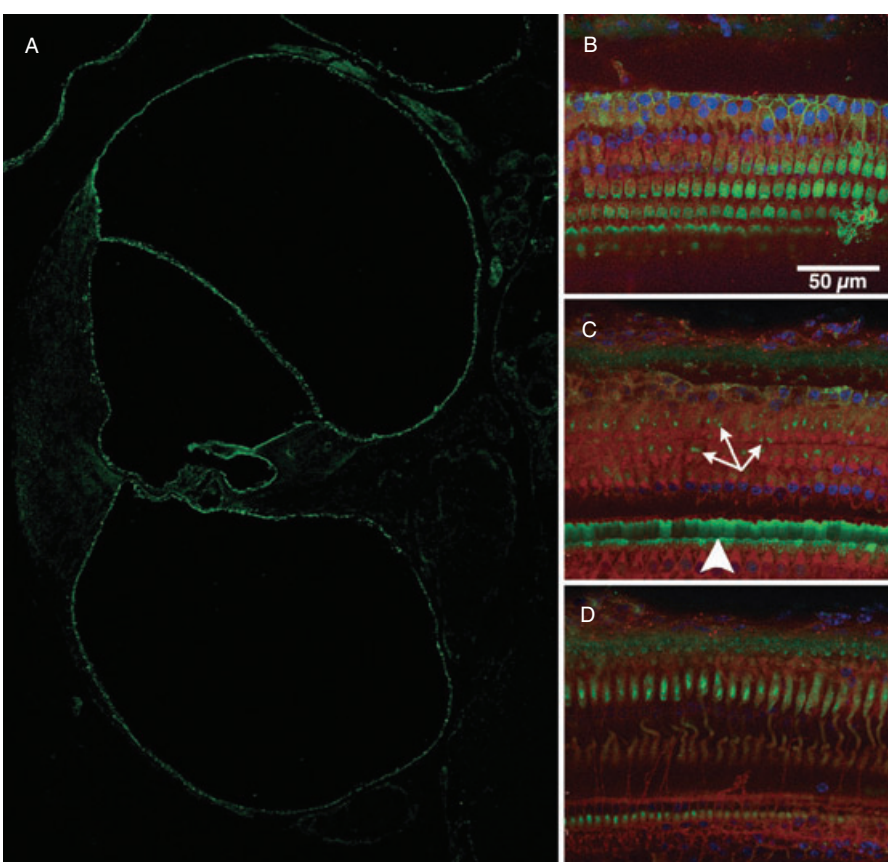

Figure 2 *ANXA1-receptor, FPR2/ALX, is expressed in guinea pig cochleae.* (A) Frozen section of a guinea pig cochlea labelled with anti-FPR2/ALX (green). (B, C, D) Optical sections of guinea pig OC showing expression of FPR2/ALX receptors (green) in the apical region of outer hair cells (B), apical region of Pillar cells (C, arrowhead) and phalangeal processes of Deiter cells (C, arrows), and basal region of Pillar cells (D). Actin structures are labelled in red with rhodamine phalloidin. ANXA1, annexin A1; OC, organ of Corti.

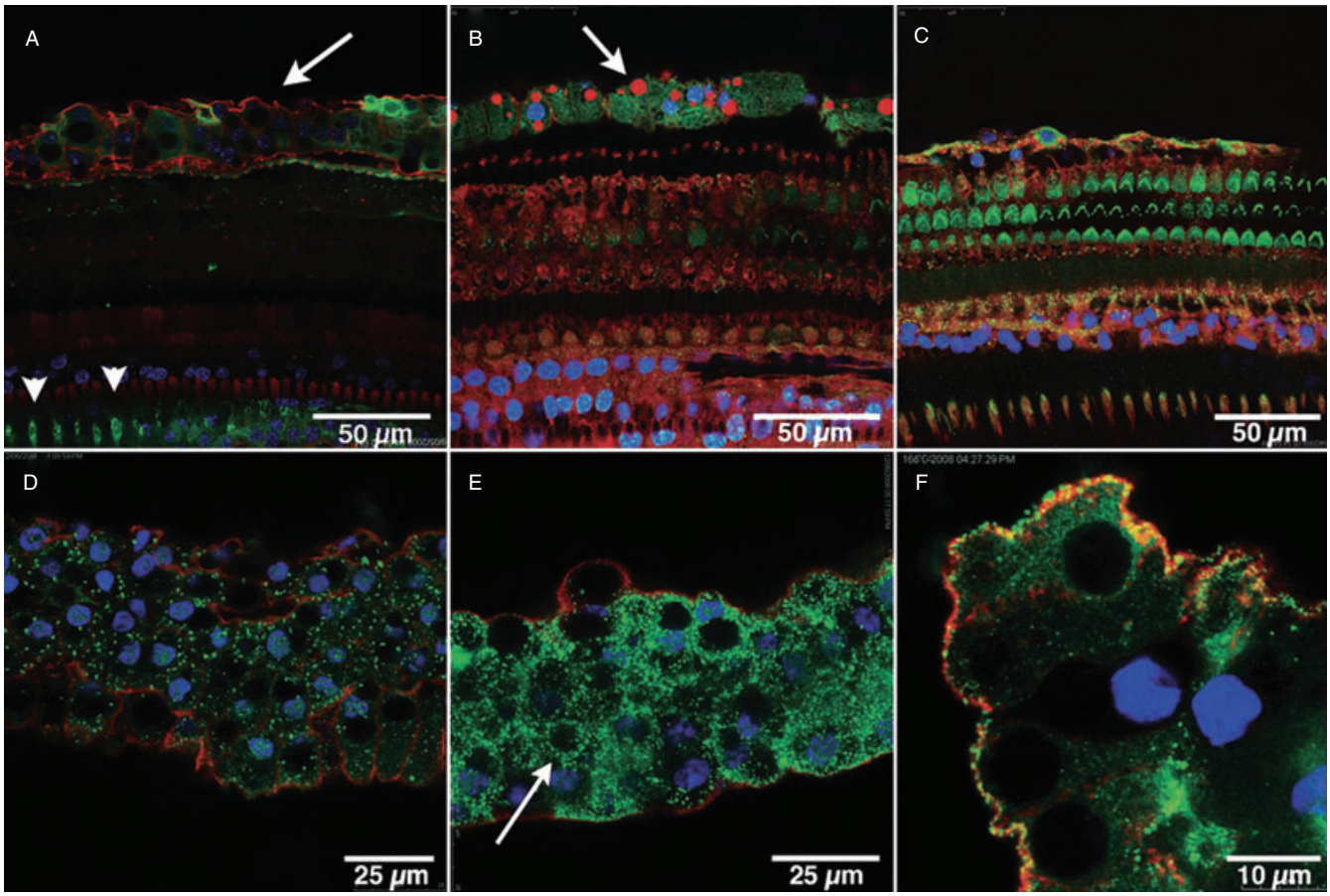

Figure 3 *Effect of dexamethasone on ANXA1 expression.* (A) Guinea pig cochlea labelled with anti-ANXA1 (green) and stained with rhodamine phalloidin (red) and DAPI (blue). ANXA1 labelling was observed in Hensen cells (arrow) and some cells in the spiral promontory (arrowheads), but not in outer and inner hair cells, Pillar cells and Deiter cells. lipid droplets were not labelled, and were observed as 'holes'. However, it should be noted that not every 'hole' in the Hensen cell cytoplasm corresponds to a lipid droplet. (B) After 5 min exposure to dexamethasone (10 nM), ANXA1 labelling (green) was observed in Hensen cell cytoplasm and also in outer hair cell cuticular plate and stereocilia bundle. Nuclei were labelled with DAPI (blue) as before but, in this case, cell membranes and lipid droplets were stained with Nile Red. Thus, lipid droplets are clearly identified as rounded red bodies inside Hensen cells (arrow). Remaining 'holes' in the cytoplasm correspond to unidentified cell structures. (C) After 45 min exposure to dexamethasone many Hensen cells look 'flattened', and lipid droplets are barely visible even with Nile Red stain. In contrast, ANXA1 labelling of outer hair cell's cuticular plate and stereocilia was strong. (D) Hensen cells isolated by microdissection, exposed to dexamethasone (10 nM, 3 min) and then fixed, show a speckled labelling with anti-ANXA1 (green). Actin cytoskeleton was labelled with rhodamine phalloidin. lipid droplets are not stained and appear as dark holes. (E) Isolated Hensen cells exposed 5 min to dexamethasone show a notable increase in ANXA1 immunoreactivity. Frequently, lipid droplet surfaces look 'decorated' with ANXA1-positive spots (arrow). (F) After 15 min exposure to dexamethasone most ANXA1-positive spots are concentrated in the apical region of Hensen cells (stronger labelling with rhodamine phalloidin than the basal and lateral regions). ANXA1, annexin A1.

DAPI (blue) and Nile Red, a lipid stain. Thus, in Figure 3B is possible to distinguish small 'holes' – corresponding to unidentified structures – next to red-labelled lipid droplets. Figure 3C, also stained with Nile Red, supports the idea that exposure to glucocorticoids resulted in flattened Hensen cells with fewer and smaller lipid droplets.

Confocal observations were complemented with microfluidic studies aimed at evaluating the amount of ANXA1 released by Hensen cells when exposed to glucocorticoids (Figure 4). Experiments with isolated Hensen cells, either not exposed to glucocorticoids or exposed to glucocorticoids for 5, 10, 15, 20, 25 or 30 min, showed that these cells released considerable amounts of ANXA1 when stimulated with glucocorticoids, and in a time-dependent manner (Figure 4A). The three glucocorticoids assayed induced a significant increase in ANXA1 release in Hensen cells in the first 5 min of exposure, followed by a rapid decrease. Consistent with our confocal observations, a noticeable 'second wave' of release was observed 15–20 min after initial exposure, followed by another quick decrease. This secondary response was more obvious in Hensen cells exposed to dexamethasone than in those exposed to hydrocortisone or prednisolone (Figure 4A). The amount of 'released' ANXA1 was inversely correlated to the amounts of 'cytoplasmic' ANXA1. As shown in Figure 4B, levels of ANXA1 in the cytoplasm of Hensen cells exposed to dexamethasone decreased abruptly in the first 3 min. A slow recovery, peaking at 15–20 min after exposure, was followed by a second decrease. In Hensen cells exposed to either hydrocortisone or prednisolone, the pattern of the response was slightly different. Like dexamethasone, hydrocortisone induced a significant decrease in cytoplasmic ANXA1 in the first 3 min of exposure, but exhibited no recovery during the

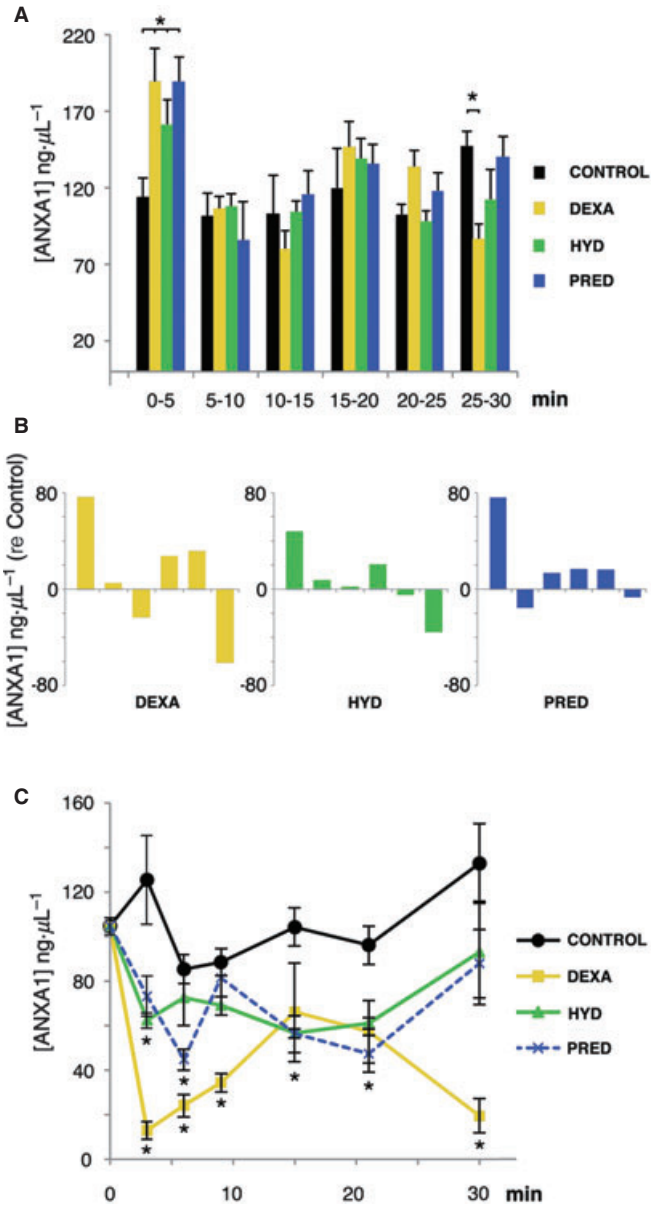

Figure 4 *Quantification of released and cytoplasmic ANXA1 in Hensen cells.* (A) Microfluidic techniques showed that the amount of ANXA1 released by isolated Hensen cells increased significantly after 5 min exposure to glucocorticoids (100 nM) with a subsequent decrease to control values. In cells exposed to dexamethasone (DEXA), a second increase in ANXA1 release after 20 min of exposure was followed by a significant decrease to levels equivalent to ~50% of control values. Hensen cells exposed to hydrocortisone (HYD) and prednisolone (PRED) showed a similar, but less evident, pattern of response. (B) Measurements of cytoplasmic ANXA1 showed an inversely correlated pattern to that of released ANXA1, with a sharp decrease during the first 3 to 6 min of exposure to glucocorticoids. The response was more evident in Hensen cells exposed to dexamethasone than to hydrocortisone or prednisolone. The data points represent the mean value of three experiments (in duplicate). Error bars represent the calculated s.e.mean. Asterisks (*) indicate $P < 0.05$. ANXA1, annexin A1.

length of our experiments. In contrast to dexamethasone and hydrocortisone, the fast decrease in cytoplasmic ANXA1 induced by prednisolone peaked approximately 6 min after exposure, followed by a fast (~3 min) recovery to normal values, with a secondary decrease and recovery (Figure 4B).

Glucocorticoids do not induce release of Lipoxin A_4 from Hensen cells

FPR2/ALX is the ANXA1 receptor but also a receptor for another very important anti-inflammatory molecule, Lipoxin A_4 (LXA_4) (Chiang *et al.*, 2006; Serhan *et al.*, 2007). Both FPR2/ALX agonists, ANXA1 and LXA_4, inhibit leukocyte migration and promote inflammatory resolution. To evaluate the potential glucocorticoid-induced release of LXA_4 by cochlear spirals, we used a specific quantitative ELISA assay. In contrast to our previous results with ANXA1, we did not find evidence of glucocorticoid-induced release of LXA_4 (Figure S2). Moreover, the concentrations of LXA_4 detected were always below the nanomolar range, several orders of magnitude lower than the observed levels of ANXA1 released by Hensen cells. These results suggest that glucocorticoids did not induce release of LXA_4 from Hensen cells or other cochlear cells included in our experiments. However, effects on cells from the spiral ligament, stria vascularis and other structures of the lateral wall of the cochlea, which are removed during the microdissection process, cannot be excluded.

Lipid droplets in Hensen cells play a role in intracellular storage for ANXA1

We used electron microscopy to investigate the effect of dexamethasone on the ultrastructure of guinea pig Hensen cells and the intracellular distribution of ANXA1. These studies showed that microvilli covering the apical surface were more numerous and more tightly packed in Hensen cells exposed to dexamethasone than in unexposed cells (Figure 5A,B). Complex blebbing of the apical membrane and the presence of many small vesicles in the cytoplasm suggested activation of an exocytic event. Anti-ANXA1 labelling was observed inside lipid droplets in Hensen cells not exposed to dexamethasone (Figure 5C), suggesting that they could be intracellular storage sites for ANXA1. In Hensen cells exposed to dexamethasone, anti-ANXA1 labelling was observed in the cell cytoplasm (Figure 5D) and lipid droplets appeared to be fragmenting and shedding (Figure 5E). Morphometric analysis showed that lipid droplets in dexamethasone-exposed Hensen cells were smaller than in untreated cells (results not shown), suggesting that glucocorticoid treatment induces a net loss in lipid droplet content.

ANXA1 is transported to the apical region of Hensen cells by a myosin IIC-based mechanism

In order to understand how ANXA1 could be rapidly transported through Hensen cell cytoplasm, we examined the cells for the presence of motor proteins. Our proteomic studies confirmed that, similar to murine Hensen cells (Donaudy *et al.*, 2004), guinea pig Hensen cells express myosin IIC (Table S1). We further validated these results with confocal microscopy studies, showing that isolated Hensen cells expressed all known non-muscle myosins II: myosins IIA, IIB and IIC. Myosin IIB was concentrated in Hensen cell nuclei, and exposure to glucocorticoids did not change its cellular distribution (Figure S3A–C). Myosin IIA labelling was found in the Hensen cell cytoplasm and the nucleus (Figure S3D). Exposure to

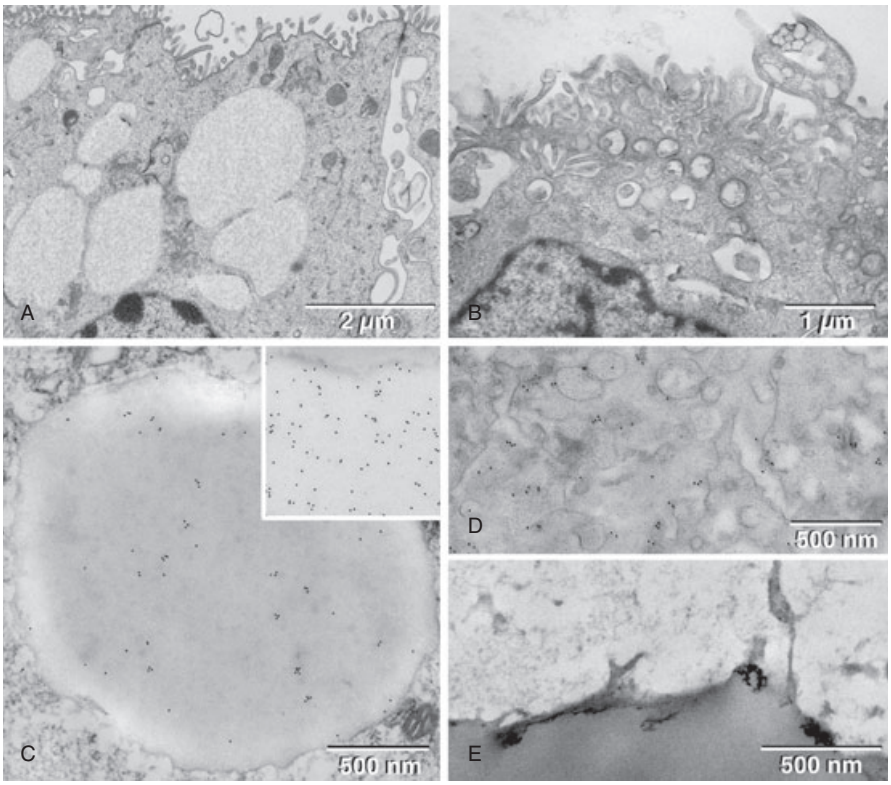

Figure 5 *Hensen cell ultrastructure: TEM of thin sections through resin-embedded Hensen cells.* (A) Strip of cells, not exposed to dexamethasone, embedded in epoxy resin. The image shows the apical region of the selected cell, with the apical surface of the cells covered in microvilli. Lipid droplets are present in the cytoplasm below the apical surface as are many recognizable subcellular organelles such as mitochondria and a Golgi complex. Although the lipid droplets are extracted, they have retained their spherical appearance. (B) Cells exposed to dexamethasone and embedded in epoxy resin using a microwave-assisted protocol. The microvilli in the apical surface are more numerous and more tightly packed on the membrane than those on cells not exposed to dexamethasone. Complex blebbing of the apical membrane is also observed and the cytoplasm contains many small vesicles. (C) Lipid droplets in a cell not exposed to dexamethasone and embedded in epoxy resin. The section was labelled with goat anti-annexin antibodies, rabbit anti-goat bridging antibodies and PAG (10 nm). Gold particles, indicating bound antibody, are present over the lipid droplet in the cell cytoplasm. Part of a lipid droplet in a cell not exposed to dexamethasone, embedded in Lowicryl HM20 resin. The section was labelled with rabbit anti-ANXA1 antibodies and PAG (10 nm). The increased amount of gold labelling may be due to the use of immuno-compatible resin. (D) Hensen cells exposed to dexamethasone and embedded in Lowicryl HM20 resin. Anti-annexin labelling is observed over many structures in the cell cytoplasm. (E) Hensen cells exposed to dexamethasone and embedded in epoxy resin. In this section, part of a lipid droplet is shown appearing to be fragmenting into the cell cytoplasm. ANXA1, annexin A1; PAG, protein A gold.

glucocorticoids induced fast translocation of a small fraction of the myosin IIA present in the cytoplasm towards the cell periphery, to not only the apical region but also the basal and lateral surfaces (Figure S3E). Labelling of myosin IIC was concentrated around vesicles in the cytoplasm of guinea pig Hensen cells, and no accumulation in the apical region of the cells was observed (Figure 6A). After stimulation with glucocorticoids, in contrast, myosin IIC labelling moved to the cell apical region (Figure 6B), co-localizing with ANXA1 labelling (Figure 6C–E). Moreover, pre-incubation of Hensen cells with blebbistatin – a myosin II inhibitor (Kovacs *et al*., 2004) – led to ANXA1 accumulation in the cell cytoplasm (Figure 7A). Microfluidic studies confirmed that blebbistatin interfered with the glucocorticoid-stimulated release of ANXA1. We found that levels of cytoplasmic ANXA1 did not increase significantly in Hensen cells after 30 min pre-incubation with blebbistatin. Continued exposure to blebbistatin results in a slight increase in cytoplasmic ANXA1, with values returning to control levels 20 min after pre-incubation (Figure 7B). In cells pre-incubated with blebbistatin (30 min) followed by exposure to glucocorticoids (with blebbistatin still present in the media), changes in the amount of cytoplasmic ANXA1 were more evident. The concentration of cytoplasmic ANXA1 increased rapidly, reaching peak values at about 15 min of exposure to glucocorticoids that were two- to threefold higher than in control cells (Figure 7B). The response was similar for the three glucocorticoids assayed, but more obvious in cells exposed to dexamethasone. Measurements of ANXA1 released by Hensen cells exposed to blebbistatin and glucocorticoids showed a wide variability and no clear pattern (Figure 7C). No significant differences with control values were found in any condition.

Release of ANXA1 by Hensen cells might involve ABC transporters

We found that, as in other systems, ANXA1 release from Hensen cells did not occur through the classical secretory pathway that involves targeting of proteins to the plasma membrane via endoplasmic reticulum and Golgi apparatus

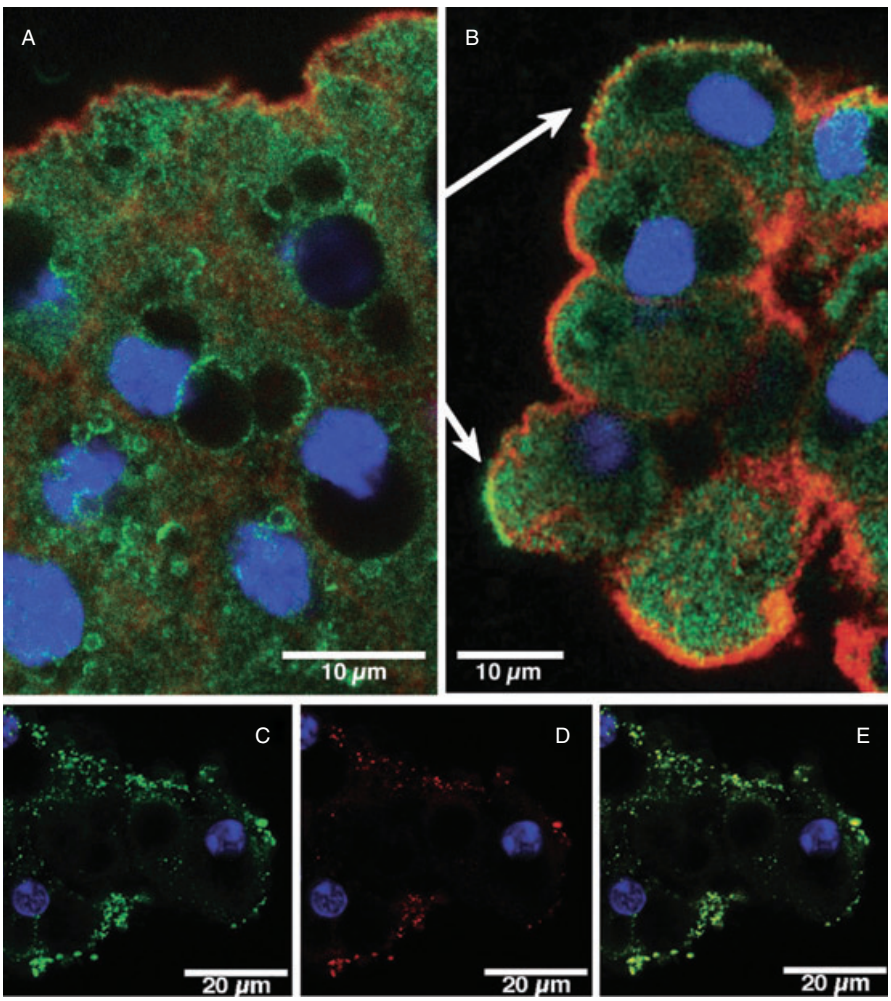

Figure 6 *Myosin IIC is expressed in guinea pig Hensen cells and translocates to the cells' apical region after stimulation with* dexamethasone. (A) Hensen cells express myosin IIC (green), and immunolabelling was frequently observed on the surface of lipid droplets and cytoplasmic vesicles. (B) After 5 min stimulation with dexamethasone (10 nM), Hensen cell cytoplasm appears filled with myosin IIC-wrapped microstructures, many of them concentrated at the apical border of the cells (arrows). Red – rhodamine-phalloidin, blue – DAPI. (C, D, E) Hensen cells stimulated with dexamethasone (10 nM, 3 min) and triple-labelled with anti-ANXA1 (green, C), anti-myosin IIC (red, D) and DAPI (blue) show co-localization of ANXA1 and myosin IIC in the microstructures concentrated at the apical region of the cells (merge, E). ANXA1, annexin A1.

(Figure 8A,B). Monensin, brefeldin A and nocodazole, three agents that interfere with the classical secretory pathway at different stages, did not prevent ANXA1 release from Hensen cells. In contrast, we found that pre-incubation with glyburide, an inhibitor of ABC transporters known to prevent ANXA1 release in other cell systems (Wein *et al.*, 2004; Omer *et al.*, 2006), diminished ANXA1 release in Hensen cells stimulated with dexamethasone (Figure 8C). Antibodies against the ABC transporter, ATP-binding cassette transporter A1 (ABCA1), labelled only a subset of guinea pig Hensen cells (Figure 8D), suggesting a role for ABCA1 in mediating ANXA1, as in other cell types (Wein *et al.*, 2004; Omer *et al.*, 2006). This result is consistent with the hypothesis that guinea pig Hensen cells are not a single, homogeneous population, but consists of several functionally different subpopulations. The mechanism by which ANXA1 release could be associated with different ABC transporters distinctly expressed in different subpopulations of guinea pig Hensen cells is currently being investigated.

ANXA1 is released by Hensen cells in response to exposure to ototoxic drugs

In order to link ANXA1 release with physiological responses to environmental insults, we exposed isolated cochleae to gentamicin or cisplatin, both well-known ototoxic agents. At concentrations known to kill OC sensory cells and initiate inflammatory responses, we found that both treatments induced release of ANXA1 from Hensen cells, and both resulted in the appearance of ANXA1 labelling on cells expressing FPR2/ALX (Figure 9A,B). However, whereas the cisplatin-induced ANXA1 release was easily detected, the effect of gentamicin was less easy to detect. Gentamicin-induced ANXA1 release was characterized by a preferential attachment of ANXA1 to the phalangeal process of the most external row of Deiter cells. In cisplatin-treated cochleae, a strong labelling with anti-ANXA1 was observed in the apical region of hair cells and Pillar cells. Optical sections of the OC reticular lamina showed that the labelling was confined to the external surface of the cells (Figure 9C), supporting the idea

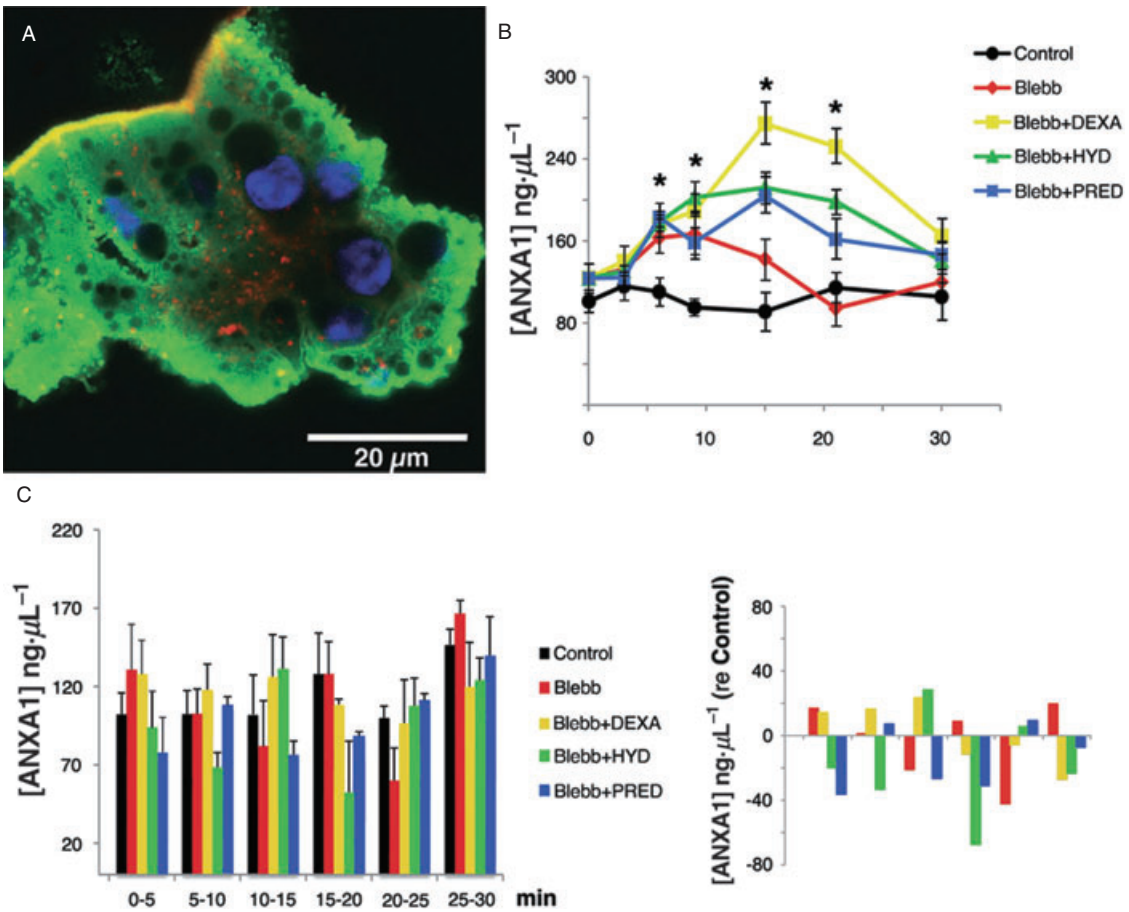

Figure 7 *The myosin II inhibitor blebbistatin interferes with the transport of ANXA1.* (A) Exposing Hensen cells pre-incubated with blebbistatin (100 μM, 30 min) to dexamethasone (DEXA; 10 nM, 15 min), increases the concentration of ANXA1 in every region of the cytoplasm and leads to a noticeable reduction in the average size of lipid droplets. ANXA1 (green), rhodamine phalloidin (red) and DAPI (blue). (B) Digital quantification with microfluidics techniques showed up to a threefold increase in the levels of ANXA1 in the cytoplasm of Hensen cells incubated with both blebbistatin (Blebb; 30 + 15 min) and dexamethasone (DEXA; 15 min). Similar changes, although less significant, were observed in Hensen cells exposed to hydrocortisone (HYD) and prednisolone (PRED). In every case, the levels of cytoplasmic ANXA1 decreased to control values after 30 min of exposure to glucocorticoids. These results suggest that blebbistatin did not interfere either with the release of ANXA1 from intracellular storages into the cytoplasm or with the release of ANXA1 to the external milieu. (C) Blebbistatin caused a >75% reduction in glucocorticoid-induced ANXA1 release in the first 5 min of exposure, with values statistically similar to those of control cells along the total duration of the experiment (30 min). Control: Hensen cells not exposed to blebbistatin or glucocorticoids; Blebbistatin: Hensen cells exposed to blebbistatin (100 μM) for 60 min (30 min pre-incubation + time of glucocorticoid exposure); DEXA, HYD and PRED: Hensen cells pre-incubated with blebbistatin (100 μM, 30 min) and then exposed to the glucocorticoids (100 nM) for the specified time in the continuous presence of blebbistatin. Data points represent the mean value of three experiments (in duplicate). Error bars represent the calculated s.e.m. Asterisks (*) indicate $P < 0.05$. ANXA1, annexin A1.

that it corresponded to ANXA1 released by Hensen cells and attached to the receptors localized in these other cochlear cells. Interestingly, in our studies with cisplatin- and dexamethasone-stimulated cells, we observed ANXA1-filled Hensen cells alternating with ANXA1-empty Hensen cells (Figure 9D). This observation is consistent with the hypothesis that, in guinea pigs, there are at least two functionally different subpopulations of Hensen cells.

Discussion

Elucidation of the mechanisms regulating inflammatory responses in the OC requires the identification of a glucocorticoid-activated mechanism able to prevent local leukocyte migration and facilitate the resolution phase of inflammation. In this work we provide evidence that, in the guinea pig OC, glucocorticoids stimulated the release of ANXA1 from cochlear Hensen cells via a myosin IIC-driven mechanism. Given that ANXA1 is known to inhibit leukocyte migration and extravasation, and to mediate in other processes associated with the resolution phase of inflammatory responses, this finding suggest that the effects of glucocorticoids in the mammalian cochlea could be mediated, at least in part, by ANXA1.

ANXA1, a protein whose synthesis and release are strongly regulated by glucocorticoids, has been described as a key modulator of both the innate and adaptive immune systems (D'Acquisto *et al.*, 2008; Perretti and D'Acquisto, 2009). ANXA1 is known to inhibit polymorphonuclear leukocytes and monocyte-macrophage extravasation, adherence and migration (Hayhoe *et al.*, 2006). It has been suggested that,

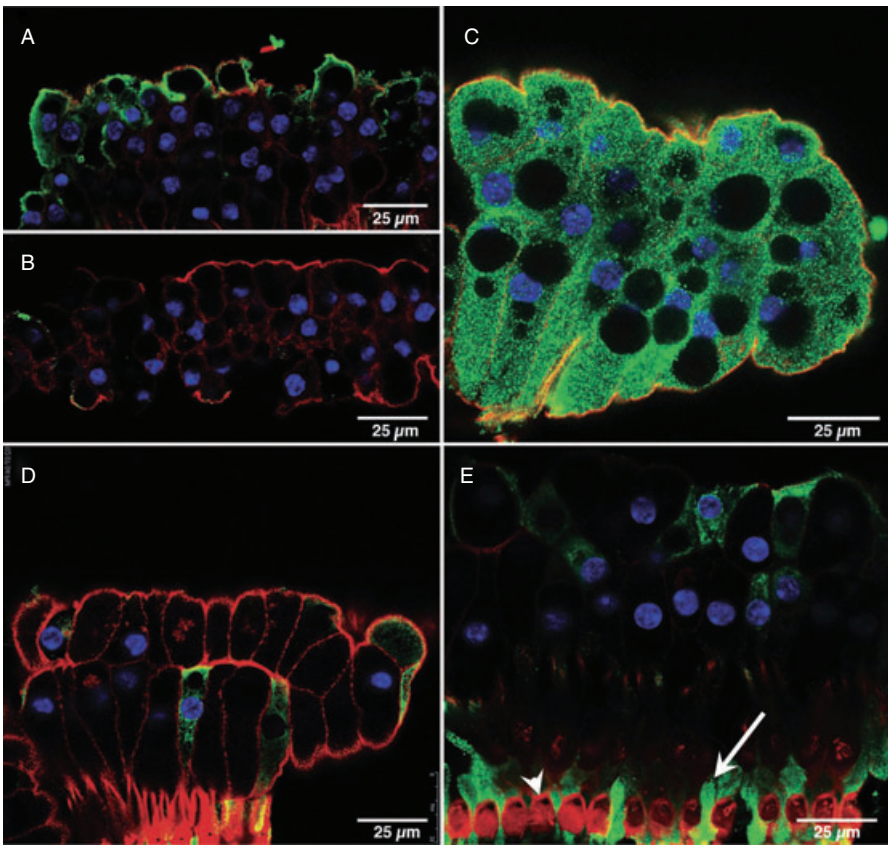

Figure 8 *ABC transporters might be involved in the release of ANXA1 by Hensen cells.* (A) In isolated Hensen cells pre-incubated with monensin (10 μM, 2 h) and then exposed to dexamethasone (10 nM, 15 min), ANXA1 labelling (green) was found in some cells only at the apical region. (B) Most of the cells in this condition, however, were no labelled at all, indicating an effective release of ANXA1 to the external milieu. Similar results were obtained with Hensen cells pre-incubated with brefeldin A (1.5 μM, 2 h) and nocodazole (3.5 μM, 2 h). (C) Pre-incubation with glyburide (100 μM, 1 h), a well-known inhibitor of ABC transporters, prevents ANXA1 release in Hensen cells stimulated with dexamethasone (10 nM, 3 min). ANXA1 labelling (green) was very strong, suggesting that glyburide does not interfere with the release of ANXA1 from intracellular stores into the Hensen cell cytoplasm. (D) Intriguingly, antibodies against ABCA1 labelled only some Hensen cells, suggesting that its expression could be restricted to some specific subset/s of guinea pig Hensen cells. (E) ABCA1 labelling was consistently strong in the apical surface of the phalangeal processes of Deiter cells (arrows), and absent in the cuticular plate of outer hair cells (arrowhead). Note that only few Hensen cells are labelled. ANXA1, annexin A1; ABCA1, ATP-binding cassette transporter A1.

following adequate stimulation, leukocytes release ANXA1 that binds to its receptor on the leukocyte plasma membrane, interfering with cellular adhesion to the endothelium, causing cell detachment, inhibiting transmigration and inducing apoptosis (Ernst *et al.*, 1990). However, in the mammalian cochlea, extravasation, cellular adhesion and infiltration of polymorphonuclear leukocytes and monocyte-macrophages are not inhibited except at the level of the SM. Thus, we speculate that inhibition of migration in the cochlea is not associated with the leukocytes themselves (e.g., ANXA1-mediated autocrine effect) but with the presence of ANXA1 in the SM and its absence in the other cochlear ducts. The specific expression of ANXA1 in epithelial cells enclosing the SM, its accumulation inside cochlear Hensen cells and the ability of these cells to release it rapidly and in large amounts, supports this hypothesis. The more generalized expression of the FPR2/ALX receptor, in contrast, could be associated with the activity of other anti-inflammatory circuitry, such as those involving lipoxins and epi-lipoxins (McMahon and Godson, 2004; Serhan *et al.*, 2008), which are known to participate in the pro-resolution process and bind to the same FPR2/ALX receptor as ANXA1. Moreover, FPR2/ALX affinity for lipoxins is higher than their affinity for ANXA1 (Perretti *et al.*, 2002; Chiang *et al.*, 2005). However, our results (Figure S2) suggest that LXA_4 may be playing a less significant role than ANXA1 in the glucocorticoid-stimulated anti-inflammatory response in the cochlear SM. Thus, it can be envisioned that inflammatory responses in the OC stimulate the recruitment of leukocytes in the spiral ligament, which then start their migration to the SM. At the SM boundary, epithelial cells could respond to attempts at disrupting the tight-junction barrier by releasing ANXA1, which would bind to the FPR2/ALX receptors present in the leukocyte plasma membrane, stopping diapedesis. In the case of strong inflammatory reactions, some leukocytes could overcome the epithelial barrier only to find themselves immersed in an endolymphatic fluid saturated with ANXA1 molecules released by Hensen cells. This second protective barrier would be enough, in most cases, to prevent inflammatory damage to the OC by inducing apoptosis of the invading leukocytes. Thus, the therapeutic effect of glucocorticoids in the cochlea would be associated with their ability to stimulate ANXA1 synthesis and release.

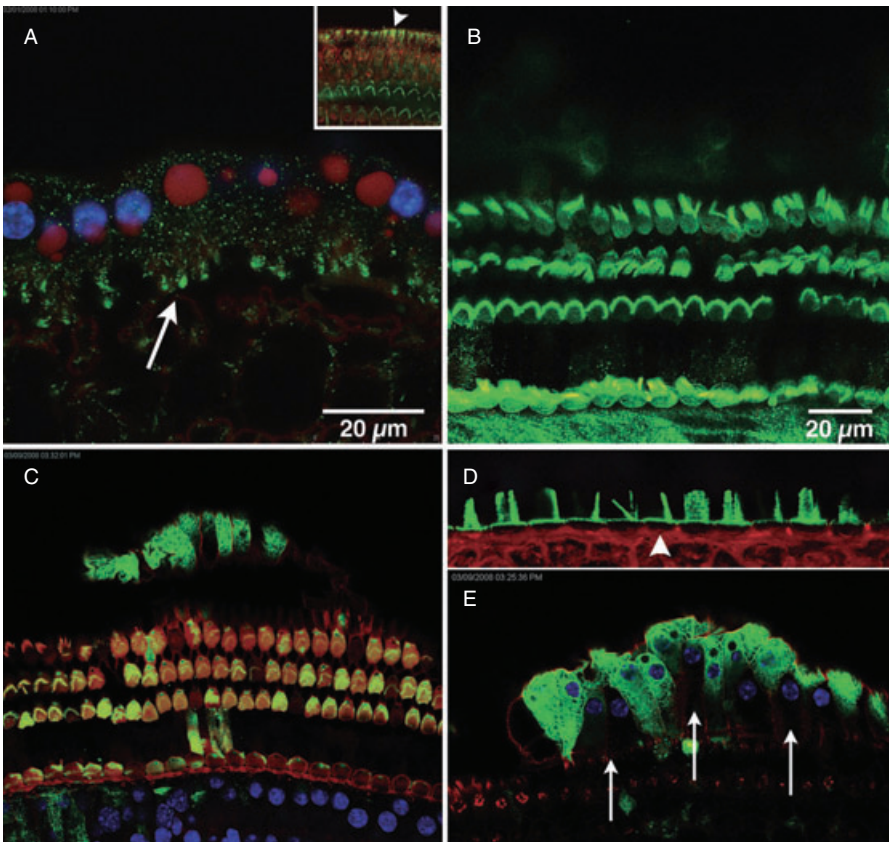

Figure 9 *Ototoxic drugs induce release of ANXA1 from Hensen cells.* (A) Exposure of guinea pig cochleae to gentamicin (100 μM, 1 h) induces a moderate release of ANXA1 (green) from Hensen cells. Interestingly, in addition to label the outer hair cell stereocilia (inset), ANXA1 was consistently concentrated inside the phalangeal processes of third row (closest to Hensen cells) Deiter cells (arrow and arrowhead in inset). Lipid droplets are stained with Nile Red. (B) Exposure to cisplatin (500 μM, 30 min), in contrast, induces a significant release of ANXA1. ANXA1 reactivity (green) in outer and inner hair cells stereocilia and cuticular plate as well as in Pillar cells makes the red labelling with rhodamine phalloidin almost invisible. (C) Guinea pig cochlea exposed to cisplatin (100 μm, 15 min). Abundant ANXA1 labelling is detected in outer hair cell stereocilia and cuticular plate. Whereas some Hensen cells are filled with ANXA1, others look empty, suggesting that they release ANXA1 at different times. (D) Detail of outer hair cells exposed *in situ* to cisplatin (100 μm, 15 min). Note absence of ANXA1 intracellular labelling (arrowhead). (E) Detail of Hensen cells exposed *in situ* to cisplatin (100 μm, 15 min). As in (C), Hensen cells labelled with anti-ANXA1 are next to unlabelled cells (arrows). ANXA1, annexin A1.

Our quantitative microfluidic data indicate that glucocorticoids induce a rapid (<5 min) release of ANXA1 from cochlear Hensen cells, which exhibits an inverse correlation with the levels of cytoplasmic ANXA1 (Figure 4). In cells exposed to dexamethasone, a significant recovery in the levels of cytoplasmic ANXA1, followed by another decrease, was also correlated with a second peak in ANXA1 release. These results may be explained in two possible ways. First, dexamethasone may induce an immediate release of ANXA1 already available in the Hensen cell cytoplasm, followed by another wave, lasting approximately 10 min, consisting of ANXA1 recovered from the cytoplasmic pool (after release from intracellular storages), translocation to the apical region of the cells, and release from the cells. The second possibility is that we have revealed the existence of at least two distinctly different Hensen cell subpopulations able to accumulate and release ANXA1 at different times. This second possibility is supported by the frequent observation of 'ANXA1-filled' next to 'ANXA1-empty' cells (Figure 9D) as well as the existence of subsets of Hensen cells expressing different ABC transporters (Figure 8D).

The identification of lipid droplets as the intracellular storage of ANXA1 and the involvement of myosin IIC in the trafficking of ANXA1 in the Hensen cell cytoplasm are two novel results. Lipid droplets, which for years were considered either benign cytoplasmic inclusions or even simple 'lipid bags', are currently viewed as *bona fide* cellular organelles (Martin and Parton, 2006; Goodman, 2008; Olofsson *et al.*, 2008; Walther and Farese, 2008). Recent studies have provided evidence that they could act as transient storage depots for proteins (Cermelli *et al.*, 2006). In most cases, however, proteins are thought to be associated with the lipid droplet surface, bound by electrostatic interactions to charged lipids or other proteins present in the monolayer that wraps the entire structure. In contrast, our ultrastructural studies seem to indicate that ANXA1 localizes preferentially inside the lipid droplets rather than at their surface. We do not yet understand how ANXA1 can be stored inside lipid droplets or the mechanism that mediates its release into the cytoplasm. As a lipid-binding protein, ANXA1 binds charged lipids in a Ca^{2+}-dependent manner, but it does not explain its sequestration in a bulk of non-polar lipids. We speculate that ANXA1 might

be stored on lipid droplets in an unfolded state so that hydrophobic residues normally buried in the protein interior are exposed and able to interact with the lipid droplet core. Unfolding proteins for storage and refolding them for release might require the assistance of chaperones, which could be associated with the lipid droplets surface and also be part of the mechanism of ANXA1 release into the cytoplasm. Ongoing proteomic studies of lipid droplets isolated from guinea pig Hensen cells in our laboratory will soon shed light on this issue. Nonetheless, their identification as ANXA1 storage site provides an important clue for the abundance of lipid droplets present in the Hensen cell cytoplasm.

It is known that lipid droplets, in many cell types from insects to humans, travel actively along microtubules (Welte *et al.*, 2005). Thus, it was suggested that many lipid droplet-associated proteins could be carried to their ultimate destination (Cermelli *et al.*, 2006). In contrast, we showed in Hensen cells the existence of an active transport of ANXA1 released from lipid droplets, stimulated by dexamethasone and mediated by non-muscle myosin IIC. The changes in ANXA1 concentration, observed in Hensen cells pre-incubated with blebbistatin and then exposed to dexamethasone (Figure 7), support the idea that myosin IIC would be involved in the mechanism of intracytoplasmic transport of ANXA1, but not with its release from intracellular storage sites into the cytoplasm or its release to the external milieu. Myosin IIC was described as the product of the gene MYO14 in 2003 (Leal *et al.*, 2003) and just 1 year later it was detected in cells surrounding the SM in mouse cochlea, with single point mutations being associated with an autosomal dominant hearing impairment in humans (DFNA4) (Donaudy *et al.*, 2004). Although these results suggested a crucial role of myosin IIC in auditory function, they provided no clues about the specific role of this protein in the mammalian inner ear (Donaudy *et al.*, 2004). Myosin IIC has a 64% identity and ~80% similarity with either myosin IIA or IIB (Golomb *et al.*, 2004), but they exhibit differential distribution and functions, as we found in Hensen cells. For example, in the neuroblastoma cell line neuro-2A, myosins IIC and IIB – but not myosin IIA – were found to be critical for driving neuronal process outgrowth, and myosins IIC and IIA – but not myosin IIB – modulated neuronal cell adhesion (Wylie and Chantler, 2008). To our knowledge, its key role in the intracellular transport of ANXA1 in cochlear Hensen cells is the first described function of myosin IIC in the auditory system.

The release of ANXA1 from Hensen cells detected in our studies could be important not only for stopping leukocyte migration into the OC, but also for facilitating the clearance of apoptotic cells in the SM. Exposure of phosphatidylserine (PS) on the outer leaflet of plasma membrane is one of the characteristic hallmarks of apoptotic cells, providing a signal for phagocytes to recognize and to ingest them as a means for clearing cellular debris. During apoptosis, ANXA1 is recruited from the cytosol and exported to the plasma membrane, where it co-localizes with PS, in a caspase-dependent manner. ANXA1 molecules in the plasma membrane of both apoptotic cells and phagocytes may act as bridging proteins, linking the phagocyte to its target cells and promoting phagocytosis (Fan *et al.*, 2004). Although the most vulnerable cells in the OC to environmental insults – the sensorimotor outer hair cells – do not express significant amounts of ANXA1, they do express the ANXA1 receptor FPR2/ALX. The presence of ANXA1 molecules released from Hensen cells and bound to receptors on the surface of outer hair cells could facilitate their identification by phagocytic cells and the removal of those dying cells also containing PS in the outer leaflet of the plasma membrane.

In conclusion, our results suggest that ANXA1 and Hensen cells could be crucial components of the cochlear immune system and the molecular and cellular targets, respectively, of glucocorticoids in the mammalian auditory organ. We provide evidence that: (i) ANXA1 is a major target of glucocorticoids in the guinea pig cochlea; (ii) several cell populations in the SM express ANXA1, but it is mainly concentrated in Hensen cells; (iii) the site of ANXA1 storage inside Hensen cells is the lipid droplet; (iv) glucocorticoids would induce shedding of ANXA1-filled micro structures from lipid droplets; and (v) these ANXA1-filled micro structures would move to the apical region of the cells by a myosin IIC-driven process, and then be released to the external milieu. We propose that ANXA1 released by Hensen cells may act by preventing leukocytes from invading the SM and, possibly, regulating the clearance of apoptotic cells. Glucocorticoids would enhance the release of ANXA1 by Hensen cells, amplifying the anti-inflammatory and cell-repair mechanisms. Our findings have identified the molecular targets and mechanisms that could be important for the future development of better clinical strategies for prevention and treatment of sudden sensorineural hearing loss.

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Conflict of interest

The authors declare that they have no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 *Isolation of Hensen cells from the guinea pig cochlea*. (A) When fresh cochlear spirals are removed from the guinea pig temporal bone Hensen cells are clearly visible, and strips with Hensen cells and a mix of outer hair cells and Deiter cells are frequently separated from the rest of the organ of Corti (arrowhead), and can be removed with fine needles. (B) Under a dissection microscope (4×), Hensen cells are easily identified by their lipid droplets, which shine like glass beads (arrowhead). Other organ of Corti cells are neatly separated (bottom half). (C) By reflux through the needle of a 50 μL Hamilton syringe, all the outer hair cells and Deiter cells separate from the strip, while Hensen cells – which are interconnected with gap-junctions – remain tightly attached and can be collected as long strings.

Figure S2 *Glucocorticoids do not induce release of LXA_4 in cochlear cells*. ELISA techniques indicate that glucocorticoids do not stimulate release of LXA_4 by cells from dissected cochlear spirals. The concentration of LXA_4 measured in our experiments was always smaller than 0.2 $pg \cdot \mu L^{-1}$, five to six orders of magnitude lower than the concentration of ANXA1 in similar samples.

Figure S3 *Expression of myosin IIA and myosin IIB in guinea pig Hensen cells*. (A) Myosin IIB immunolabelling (green) localizes mostly in Hensen cells nuclei. Actin was stained with rhodamine phalloidin (red). (B) Same as in (A), but with DAPI confirming nuclear localization of myosin IIB. (C) Exposure to dexamethasone (DEXA; 1 nM, 15 min) did not induce significant redistribution of myosin IIB labelling. (D) Myosin IIA is abundantly expressed in guinea pig Hensen cells, mainly at the cytoplasm. (E) Exposure to dexamethasone (DEXA; 1 nM, 15 min) induced partial translocation of myosin IIA to the cell periphery, mostly at the apical but also at the basolateral region (arrowheads).

Table S1 *Hensen cell proteome identified by SDS-PAGE followed by LCESI-MS/MS*. Searches were performed with enzyme constraints and a static modification of 57.05 Da for carboxyamidomethylation on the cysteine residue of each peptide. Two missed cleavages were allowed. The parent ion mass tolerance and the fragment ion mass tolerance were 1.4 and 0 respectively. The identification criteria was: (i) Xcorr $\geq$1.5 for +1 charged peptides; (ii) Xcorr $\geq$2.0 for +2 charged peptides; (iii) Xcorr $\geq$2.5 for +3 charged peptides; and (iv) protein probability score $\leq$0.001. Proteins with two or more spectra after manual validation were accepted.

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