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Calcineurin Activation Contributes to Noise-Induced Hearing Loss

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Acoustic overstimulation increases Ca^{2+} concentration in auditory hair cells. Because calcineurin is known to activate cell death pathways and is controlled by Ca^{2+} and calmodulin, this study assessed the role of calcineurin in auditory hair cell death in guinea pigs after intense noise exposure. Immediately after noise exposure (4-kHz octave band, 120 dB, for 5 hr), a population of hair cells exhibited calcineurin immunoreactivity at the cuticular plate, with a decreasing number of positive-stained cells on Days 1–3. By Day 7, the levels of calcineurin immunoreactivity had diminished to near control, non-noise exposed values, concomitant with an increasing loss of hair cells. Staining of hair cell nuclei with propidium iodide (PI), restricted to calcineurin-immunopositive cells, indicated breakdown of cell membranes symptomatic of incipient cell death. The local application of the calcineurin inhibitors, FK506 and cyclosporin A, reduced the level of noise-induced auditory brain stem response threshold shift and hair cell death, indicating that calcineurin is a factor in noise-induced hearing loss. The results suggest that calcineurin inhibitors are of potential therapeutic value for long-term protection of the morphologic integrity and function of the organ of Corti against noise trauma.

Key words: immunocytochemistry; hair cells; FK506; cyclosporin A; guinea pig

Overexposure to intense sound can cause permanent damage to the inner ear and hearing loss (Hawkins et al., 1976). The structural changes underlying permanent noise-induced hearing loss (NIHL) include loss of the sensory hair cells of the inner ear and damage to their stereocilia. Because hair cells do not regenerate in the mammalian cochlea under normal conditions, loss of receptor cells and the associated hearing loss are irreversible and cumulative. Cochlear neurons degenerate secondarily, after the loss of sensory hair cells, suggesting that hair cells should be the primary targets for therapeutic interventions aiming to prevent hearing loss. In addition to direct noise-induced mechanical destruction of cells of the inner ear, oxidative stress associated with the formation of free radicals (Yamane et al., 1995; Ohlemiller et al., 1999; Ohinata et al., 2000) and excitotoxicity (Puel et al., 1998) are factors contributing to the pathogenesis of hair cell and hearing loss after noise trauma. Evidence from various cell lines and in vivo neuronal and nonneuronal model systems shows that cell death can be induced by oxidative stress and excitotoxicity through Ca^{2+} overload (Mattson, 2000; Aarts et al., 2003; Scorrano et al., 2003). Intracellular Ca^{2+} overload can cause cytotoxicity and trigger apoptotic and necrotic cell death pathways (Orrenius et al., 2003).

Acoustic overstimulation increases the $[Ca^{2+}]_i$ concentration in auditory hair cells (Fridberger et al., 1998; Oliver et al., 2001). Elevated $[Ca^{2+}]_i$ has been implicated in the impairment of hair cell function, may initiate hair cell damage after noise exposure, and Ca^{2+} blockers can attenuate NIHL (Heinrich et al., 1999). There are a number of pathways through which Ca^{2+} may contribute to cell death, involving activation of nitric oxide synthase (NOS) (Huang et al., 2002), phospholipase A2 (Caro and Cederbaum, 2003), proteases (Kim et al., 2002), and calcineurin (Jayaraman and Marks, 2000; Orrenius et al., 2003). The role of calcineurin as a factor in NIHL has not yet been assessed and is the focus of this report. Calcineurin belongs to the family of Ca^{2+}/calmodulin-dependent protein phosphatases, protein phosphatase 2B. Calcineurin is activated by binding of Ca^{2+}/calmodulin and is the only protein phosphatase that is regulated by a second messenger, Ca^{2+} (Morioka et al., 1999). Calcineurin is involved in immune system responses (Kincaid, 1995), cardiac hypertrophy (Sussman et al., 1998), neuronal and muscle development (Schiaffino and Serrano, 2002), the second messenger cAMP pathway (Antoni et al., 1998), Na/K ion transportation (Tumlin, 1997), memory (Mansuy et al., 1998; Winder et al., 1998), and cell

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death (Asai et al., 1999; Wang et al., 1999). FK506 and cyclosporin A are calcineurin antagonists that are used clinically in humans as immunosuppressors. They form complexes with respective binding proteins, FKBP12 (FK506-binding protein) and cyclophilin A. These complexes bind to a common composite surface made up of residues from the catalytic subunit of calcineurin (Ke and Huai, 2003), and in turn inhibit calcineurin activity.

We hypothesize that calcineurin activation plays a role in NIHL. To test this hypothesis in the present study, we: (1) assessed expression of calcineurin immunocytochemically in hair cells after noise exposure; (2) evaluated the relationship between calcineurin immunoreactivity and cell viability, using propidium iodide (PI) to assess incipient cell death; and (3) measured the effectiveness of the calcineurin inhibitors FK506 and cyclosporin A to attenuate noise trauma, as measured by noise-induced threshold shift in the auditory brain stem response and induced hair cell death. To the best of our knowledge, the present work is the first evidence indicating that calcineurin can be a significant factor in NIHL.

MATERIALS AND METHODS

Animals

Pigmented male guinea pigs (200–400 g, 2 weeks–4 weeks; Elm Hill Breeding Labs, Chelmsford, MA) were used in this study. The experimental protocol was approved by the Animal Care and Use Committee at the University of Michigan and conforms to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Noise Exposure

Animals were exposed to one octave band noise (OBN) centered at 4 kHz, at 120 dB SPL, for 5 hr in a ventilated sound exposure chamber. The sound chamber was fitted with speakers (Model 2450H; JBL) driven by a noise generator (ME 60 Micrographic equalizer; Rane) and power amplifier (HCA-1000 high current power amplifier; Parasound Products). Sound levels were calibrated (Type 2203 precision sound level meter, Type 4134 microphone; Bruel and Kjar Instruments) at multiple locations within the sound chamber to ensure uniformity of the stimulus, using a fast Fourier transform network analyzer with a linear scale. The stimulus intensity varied by a maximum of 3 dB across measured sites within the exposure chamber. During noise exposure, noise levels were monitored using a sound level meter, a preamplifier, and a condenser microphone. The microphone was positioned within the cage at the level of the animal's head.

Immunocytochemistry for Calcineurin and Rhodamine Phalloidin

Immunocytochemistry was carried out in animals not receiving any treatment and in animals at 4 time points (immediate, Day 1, 3, and 7) after noise exposure ($n = 3$ each). Animals were anesthetized deeply with ketamine (40 mg/kg, intramuscularly) and xylazine (10 mg/kg, intramuscularly) and the temporal bones removed and transferred into 4% paraformaldehyde in phosphate-buffered saline (PBS). Under a dissecting microscope, the round and oval windows and the bone near the apex were opened, followed by gentle local perfusion from the apex. The tissue was kept in the fixative for overnight. After PBS rinses and removal of the bony capsule and the lateral wall tissues, the modiolar core including the organ of Corti was removed from the temporal bone. Tissue was incubated in blocking solution consisting of 3% normal goat serum (Antibodies Incorporated, Davis, CA) in 0.3% Triton X-100 in PBS for 30 min. The organ of Corti was stained with monoclonal anti-calcineurin antibody (1:100; BD Bioscience Pharmingen, San Diego, CA) overnight at 4°C. Samples were washed three times in PBS and then incubated for 1 hr in a 1:200 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR). Tissue were then washed three times with PBS and incubated with a 1:100 dilution of rhodamine phalloidin (Molecular Probes) for 40 min to outline hair cells and their stereocilia. After three PBS rinses, the organ of Corti was dissected and mounted as a surface preparation. Immunolabeling was visualized using Olympus FV-500 confocal microscope. Sections for immunocytochemical assessment were taken from an area approximately 10 mm from the apex.

PI Staining and Immunocytochemistry for Calcineurin and Coumarin Phallacidin

Animals were sacrificed immediately after noise exposure ($n = 3$) under deep xylazine and ketamine anesthesia. Temporal bones were removed, transferred into 5 μg/ml PI (Molecular Probes) in Hanks' balanced salt solution (HBSS) (Invitrogen), and perfused carefully with PI as above. The tissues were incubated with PI solution for 15 min, washed with gentle local perfusion of HBSS, fixed by local perfusion of 4% paraformaldehyde in PBS, and then incubated overnight. The same regimen of immunocytochemistry was followed as before, except that coumarin phallacidin was used instead of rhodamine phalloidin.

FK506 and Cyclosporin A Cochlear Infusions

Additional experiments were designed to determine whether local application of the calcineurin inhibitors FK506, at concentration of 0.01 ($n = 6$), 0.1 ($n = 6$), 1 ($n = 8$), or 10 ($n = 8$) μg/ml, or cyclosporin A (10 μg/ml; $n = 7$), into the scala tympani could rescue the cochlea from noise-induced damage. FK506, cyclosporin A, or artificial perilymph (AP) ($n = 8$) was infused into the left cochlea via an osmotic pump/cannula system over 14 days initiated 4 days before noise exposure and continuing until sacrifice.

Alzet mini-osmotic pumps (model 2002, 0.5 μl/hr for 14 days; Alza Corp., Palo Alto, CA) were filled with treatment solution FK506 (Prograf; Fujisawa Healthcare, Deerfield, IL) or cyclosporin A (Sandimmune; Novartis, Basel, Switzerland) diluted in AP (sterile normal Ringer's solution, pH 7.34, osmotic pressure 285 mOsm/L, NaCl 145 mM, KCl 2.7 mM, $MgSO_4$ 2.0 mM, $CaCl_2$ 1.2 mM, and HEPES, $C_8H_{18}N_2O_4S$, 5.0 mM). Animals were anesthetized with ketamine/xylazine, and surgical procedures for cochlea microcannulation and osmotic pump implantation were carried out as described in Prieskorn and Miller (2000).

To allow time for recovery from surgery, the pump/cannula was implanted 4 days before noise exposure. Auditory brainstem response (ABR) was assessed bilaterally 3–7 days before surgery, to assure normal hearing, and on Day 3 after implant surgery, to assure the absence of surgical-induced inner ear damage. Those subjects showing no surgery-induced threshold shift were exposed to noise. ABRs were reassessed 10 days after noise exposure. On the following day the animal was sacrificed, and the middle ear space, cannula tip placement, and cannula/pump connection were examined carefully, after which both cochleae were removed. Any animal with postsurgical hearing loss, middle ear problem, or cannula/pump malfunction was excluded from the study. Ears were then processed for histologic assessment.

Auditory Brainstem Responses

To establish baseline auditory thresholds, animals were anesthetized deeply with xylazine and ketamine. A differential active needle electrode was placed subcutaneously below the test ear, a reference electrode at the vertex, and a ground electrode below the contralateral ear. The sound stimulus consisted of 15-msec tone bursts, with a rise-fall time of 1 msec at frequencies of 4, 8, and 16 kHz, generated by a Fordham Audio Generator (Model AG-298; Fordham Radio Supply, Hauppauge, NY). The stimuli were presented to the external auditory meatus in a closed acoustic system through a tube connected to a transducer (Beyer DT-48; Beyer Dynamic, Farmingdale, NY). Auditory brain stem responses were elicited by tone presentations delivered at 10/sec. The responses to 1,024 presentations were averaged using a Tucker-David data aquisition system with computer and custom software. The resulting ABR wave traces were displayed on an oscilloscope and visually evaluated. Hearing threshold was defined as the lowest stimulus intensity at which a repeatable wave form (with clearly identifiable peaks 3 and 4) was obtained.

Cytocochleogram

After fixation with 4% paraformaldehyde in PBS, the modiolar core including the organ of Corti was removed from the temporal bone. After permeabilization with 0.3% Triton X-100 for 30 min, the organ of Corti was stained for F-actin with a 1:100 dilution of rhodamine phalloidin for 40 min to outline hair cells and their stereocilia for a quantitative assessment. After being washed with PBS, the organ of Corti was dissected and mounted as surface preparations. Tissues were observed under fluorescence microscopy and the number of missing inner and outer hair cells (IHC and OHC, respectively) was counted from the apex to the base in 0.19-mm sections. Missing hair cells were apparent as dark spots or the typical phalangeal scar of supporting cells. Counting was begun 0.95 mm from the apex, omitting the irregular apical part of the cochlear spiral. Percentages of hair cell loss in each 0.19-mm length of tissue were plotted along the cochlear length as a cytocochleogram. The region of greatest damage from 4-kHz centered OBN, as demonstrated in this and previous studies (Shoji et al., 2000; Yamashita et al., 2003, 2004), was 8.93–13.11 mm from the apex. Differences in this region were evaluated for statistical significance.

Statistical Analysis

All values are presented as mean ± standard error of the mean (SEM). Differences among the different groups were evaluated using one-way analysis of variance (ANOVA) followed by Student-Newman-Keul's as a post-hoc test. Additionally, differences between the treated (left) ears and untreated (right) ears in each group were assessed using an unpaired Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Noise Increases Calcineurin Immunoreactivity in Outer Hair Cells (OHCs)

Immunohistochemistry was used to assess hair cell loss and expression of calcineurin after noise exposure. For the assessment of calcineurin, measures were taken at five time points: no noise exposure, immediately after exposure, and 1, 3, and 7 days after exposure.

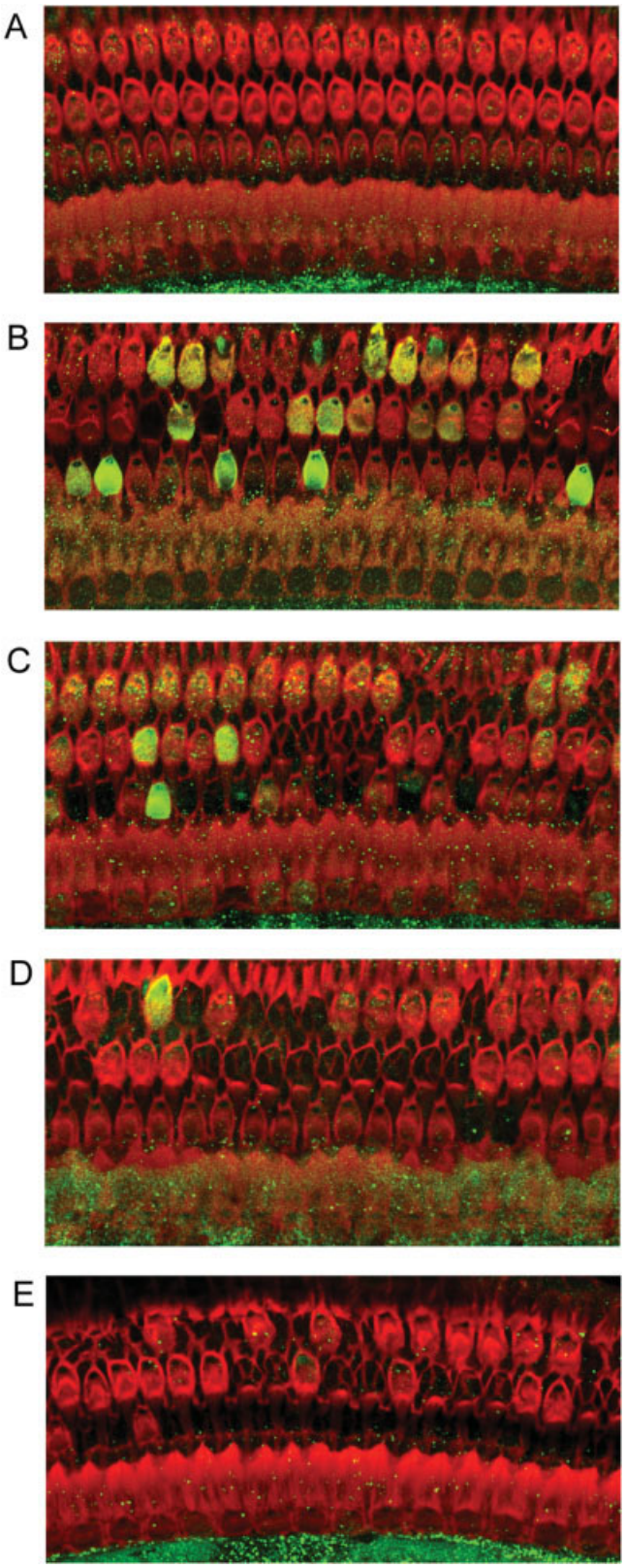
As shown in Figure 1A, in a representative surface preparation of unexposed control tissue the organ of Corti showed normal cellular architecture and no calcineurin staining in hair cells. After noise exposure, varying degrees of hair cell loss and calcineurin immunoreactivity were present in outer hair cells, depending on the time after exposure. Tissue from animals sacrificed immediately after noise exposure (Fig. 1B) showed the greatest numbers of calcineurin-immunopositive hair cells, with decreasing numbers of positive-staining cell on Days 1–3 (Fig. 1C,D). By Day 7 (Fig. 1E), the levels of calcineurin immunoreactivity had diminished to near control, non-noise exposed values. We observed no calcineurin staining in inner hair cells at any time points, and no specific staining in other types of cells on the surface preparation. Sections were taken approximately 10 mm from the apex.

Calcineurin Is Associated With Dying Hair Cells

To determine whether calcineurin-immunopositive hair cells were in fact dying, PI staining of hair cell nuclei was used to indicate cell membrane breakdown, symptomatic of incipient cell death. Figure 2 (A–I) shows a representative surface preparation of cochlear tissue taken immediately after noise exposure showing observations at nine depths of focus, descending from the cuticular plate (Fig. 2A) to the nuclear region (Fig. 2I), with a distance of 1 μm between images. After noise exposure, calcineurin seems concentrated at the cuticular plate. PI staining is restricted to calcineurin-positive cells (Fig. 2J). Some calcineurin-immunopositive hair cells demonstrated condensed nuclei (Fig. 2I, arrow), and some showed swollen nuclei (Fig. 2I, arrowhead), indicating that calcineurin is related to both apoptosis and necrosis.

Calcineurin Inhibitors FK506 and Cyclosporin A Reduce NIHL

Of 43 guinea pigs that underwent left ear surgery for microcannulation/pump-implantation, 9 animals were excluded from the study due to postsurgical hearing loss (4), middle ear infection (4), or a disconnected pump (1). In total, 34 subjects were used for data analysis: 6 received



AP; 4 FK506 (0.01 μg/ml); 5 FK506 (0.1 μg/ml); 7 FK506 (1 μg/ml); 7 FK506 (10 μg/ml); and 5 cyclosporin A (10 μg/ml). Figure 3A illustrates ABR threshold shifts for each group at each frequency on Day 10 after noise exposure for the treated side (left ears). Treatment with FK506 demonstrated a dose-dependent attenuation of noise-induced ABR threshold shifts. Ears treated with 1 or 10 μg/ml FK506 or 10 μg/ml cyclosporin A showed significantly smaller threshold shifts at each frequency compared to that of the AP-treated ears. Concentrations of 0.01 and 0.1 μg/ml of FK506 were insufficient to produce a statistically significant attenuation. The efficacy of FK506 was saturated at 1 μg/ml for all frequencies. The left cochleae, perfused with 1 (Fig. 3E) or 10 (Fig. 3F) μg/ml FK506 or 10 μg/ml cyclosporin A (Fig. 3G), demonstrated a significantly smaller ABR threshold shift than the untreated right cochleae. In contrast, left cochleae perfused with AP (Fig. 3B), 0.01 μg/ml FK506 (Fig. 3C), or 0.1 μg/ml FK506 (Fig. 3D) showed no significant differences compared to the untreated right cochleae.

Calcineurin Inhibitors FK506 and Cyclosporin A Reduce Noise-Induced Hair Cell Loss

The number of preserved and missing hair cells was assessed at 10 days after noise exposure, after final ABR recordings. Cytocochleograms (Fig. 4) illustrate the average percentage of missing OHCs along the organ of Corti for each group. Hair cell loss was significantly reduced by 1 and 10 μg/ml of FK506 and 10 μg/ml cyclosporin A, whereas 0.01 and 0.1 μg/ml FK506 showed a statistically insignificant tendency to reduce hair cell loss (Fig. 4A–E). OHC damage was reduced by 82% (1 μg/ml FK506), 82% (10 μg/ml FK506), and 78% (10 μg/ml cyclosporin A) compared to that in AP controls.

Figure 5 shows the mean percentage (± SEM) missing OHC at 8.93–13.11 mm from the apex. Results from the groups treated with FK506 at 1 and 10 μg/ml and 10 μg/ml cyclosporin A were significantly different from those of the AP treated group (Fig. 5A). FK506 demonstrated a clear dose-dependent efficacy in the attenuation of noise-induced OHC loss. With increasing concentration of FK506, OHC loss was reduced from approximately 60% observed with AP to a 10% loss with 10 μg/ml FK506. Although left cochleae perfused with 1

Fig. 1. Hair cell loss and calcineurin immunoreactivity in noise exposed cochlea. Confocal images of anti-calcineurin (green) and rhodamin phalloidin (red) double-labeled surface preparation. Sections were taken approximately 10 mm from apex, which is the area known to be damaged by 4-kHz OBN. Tissue were taken at time intervals from no noise exposure (**A**), to immediately after exposure (**B**), and 1 (**C**), 3 (**D**), and 7 days (**E**) after noise exposure. No calcineurin staining was seen in normal hair cells of nonexposed cochlea (A). Immediately after noise exposure, green calcineurin immunoreactivity was present in some OHCs (B), with decreasing numbers of positive-staining cell on Days 1–3 (C, D). On Day 7 (E), the levels of calcineurin immunoreactivity were most diminished, concomitant with an increasing loss of hair cells.

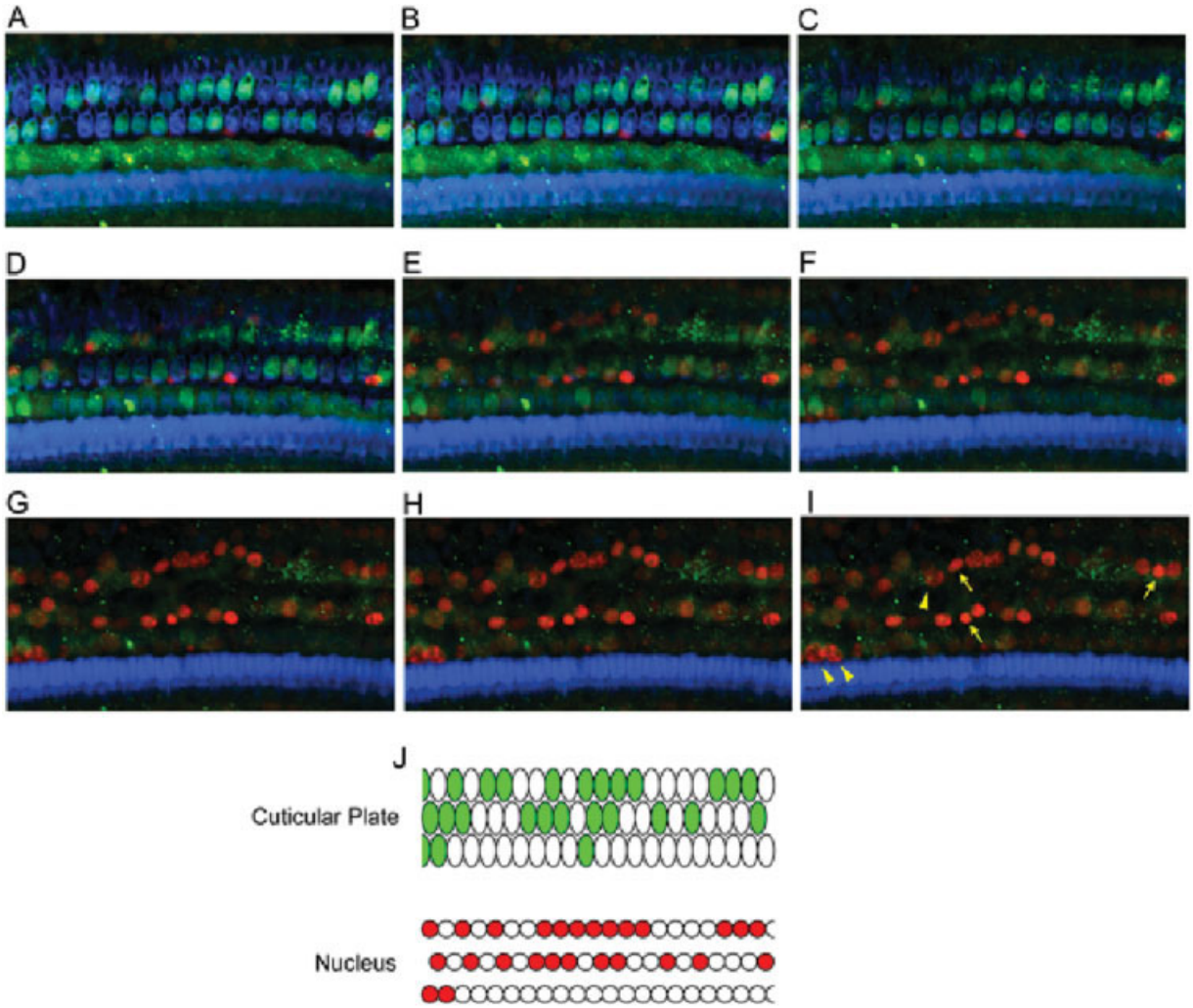


Fig. 2. Calcineurin is associated with dying OHCs. One surface preparation of cochlea tissue taken immediately after noise exposure and triple labeled for calcineurin (green), coumarin phallacidin (blue), and PI (red) is shown at nine depths of focus (**A–I**), descending from the cuticular plate (A) to the nuclear region (I) in increments of 1 μm. Note that calcineurin seemed to concentrate at the cuticular plate region, and that OHCs exhibiting calcineurin immunoreactivity at the cuticular plate show PI penetration of nuclei, in which some are condensed (arrow) and others are swollen (arrowhead). **J:** Simplified diagram of the cuticular and nucleus regions of this section.

(Fig. 5E) or 10 μg/ml FK506 (Fig. 5F) or 10 μg/ml cyclosporin A (Fig. 5G) showed significantly smaller OHC loss than did the contralateral untreated cochleae, those cochleae treated with AP (Fig. 5B), 0.01 μg/ml (Fig. 5C), and 0.1 μg/ml FK506 (Fig. 5D) showed no significant differences between treated and untreated cochleae.

DISCUSSION

After noise exposure, calcineurin immunoreactivity was observed in hair cells in the region approximately 10 mm from the apex of the cochlea, which is the region maximally damaged by 4 kHz OBN (Tsuji and Liberman, 1997; Ohinata et al., 2000; Shoji et al., 2000). Tissue from animals sacrificed immediately after noise exposure showed the greatest number of calcineurin-immunopositive hair cells, indicating that calcineurin is an early response to noise stress. On Days 1–3, the number of calcineurin-immunopositive hair cells had decreased, and by Day 7, the level of calcineurin immunoreactivity in hair cells had diminished to near control levels. Because the rate of hair cell loss was the greatest immediately after noise exposure and decreased over succeeding days (Yamashita et al., 2003, 2004), the distribution of calcineurin-immunopositive hair cells may parallel the rate of hair cell loss, i.e., those cells expressing calcineurin may die. Careful assessment at a range of depths from the cuticular plate to the nuclear region indicated that calcineurin concentrated at the cuticular plate. It is not clear if the calcineurin immunoreactivity results from expression or localization of calcineurin. Expression of calcineurin is induced by insulin-like growth factor (IGF)-I (Musaro et al., 1999;

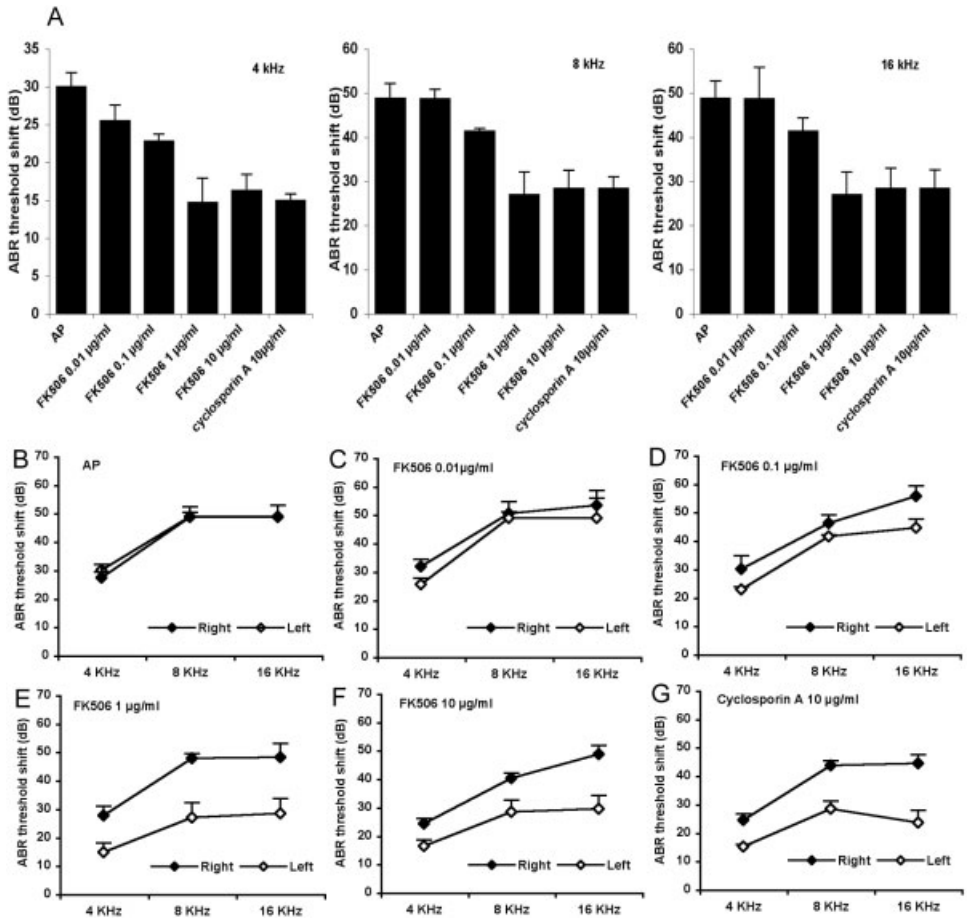


Fig. 3. Local delivery of the calcineurin inhibitor FK506 and cyclosporin A into the cochlea attenuates NIHL. Hearing threshold shifts were assessed by auditory brainstem testing at 4, 8, and 16 kHz, 10 days after noise exposure of AP- ($n = 6$), FK506- ($n = 4$ at 0.01, $n = 5$ at 0.1, $n = 7$ at 1, and $n = 7$ at 10 μg/ml), and cyclosporin A-treated ($n = 5$) guinea pigs. Changes in hearing thresholds are expressed as mean values ± SEM. Note the dose-dependent efficacy of FK506. **A:** The average threshold shifts in AP-treated animals were significantly greater than were those of 1 and 10 μg/ml FK506-and 10 μg/ml cyclosporin A-treated ears, but there were no significant effects for 0.01 and 0.1 μg/ml FK506. **B–G:** Comparisons: AP- (B), 0.01 (C), 0.1 (D), 1 (E), and 10 μg/ml FK506- (F) and 10 μg/ml cyclosporin A- (G) treated left cochleae (white diamond) with contralateral untreated right cochleae (black diamond) in the same animals exposed to acoustic trauma. Left cochleae treated with 1 and 10 μg/ml FK506 and 10 μg/ml cyclosporin A showed significantly smaller ABR threshold shifts than did the contralateral untreated cochleae. Cochleae infused with AP, 0.01 μg/ml FK506, or 0.1 μg/ml FK506, however, showed no significant differences compared to the contralateral untreated cochlea.

Gooch et al., 2001) or tumor growth factor (TGF)β (Gooch et al., 2004), and localization of calcineurin is determined by various anchoring proteins regulating calcineurin physiologic activity (Dodge and Scott, 2003). At postsynaptic dendrites, calcineurin interacts with A kinase-anchoring protein (AKAP79/150) and is localized to the cell membrane (Oliveria et al., 2003). This complex in turn modulates actin remodeling (Gomez et al., 2002). It is not known if these proteins are present in hair cells or if they are influenced by noise.

Supporting the suggestion that cells expressing calcineurin immunoreactivity may die, hair cells demonstrating calcineurin immunoreactivity also showed PI labeling. Because PI is membrane impermeable and generally excluded from viable cells, PI is used to identify nuclei of dying cells in a population. PI staining was restricted to calcineurin-immunopositive hair cells, indicating that calcineurin immunoreactivity is associated with hair cell death. Nuclear condensation and shrinkage are typical morphologies of apoptosis, whereas nuclear swelling is a feature of necrosis. After noise exposure, both apoptotic and necrotic nuclei were observed in hair cells demonstrating co-labeling for calcineurin and PI. These findings support previous studies reporting that noise-induced hair cell death involves mechanisms of both apoptosis and necrosis (Hu et al., 2002; Wang et al., 2002).

There are a variety of biochemical cascades induced by calcineurin that may lead to apoptotic and necrotic cell death. Calcineurin dephosphorylates NOS (Dawson et al., 1993) leading to nitric oxide-induced cell death pathways. Calcineurin also dephosphorylates the Bcl-2 family protein BAD (Wang et al., 1999), which activates apoptotic death pathways; and calcineurin may inactivate Bcl-2 (Erin et al., 2003a,b), an antiapoptotic protein. Calcineurin is cleaved directly by calpain and overexpression of cleaved calcineurin can induce caspase activity and neuronal cell death (Wu et al., 2004). Moreover, calcineurin is also involved in oxidative stress-induced cell death (See and Loeffler, 2001; Mbebi et al., 2002). Most of these pathways require the activation of calcineurin. It was therefore of interest to determine whether the calcineurin inhibitors would protect hair cells against acoustic trauma.

The observation that the calcineurin inhibitors FK506 and cyclosporin A attenuate noise-induced hearing loss and hair cell loss indicates that the activation of calcineurin is important in NIHL. Although a contralateral effect has been reported with high concentrations of protective agent in other studies involving local application of

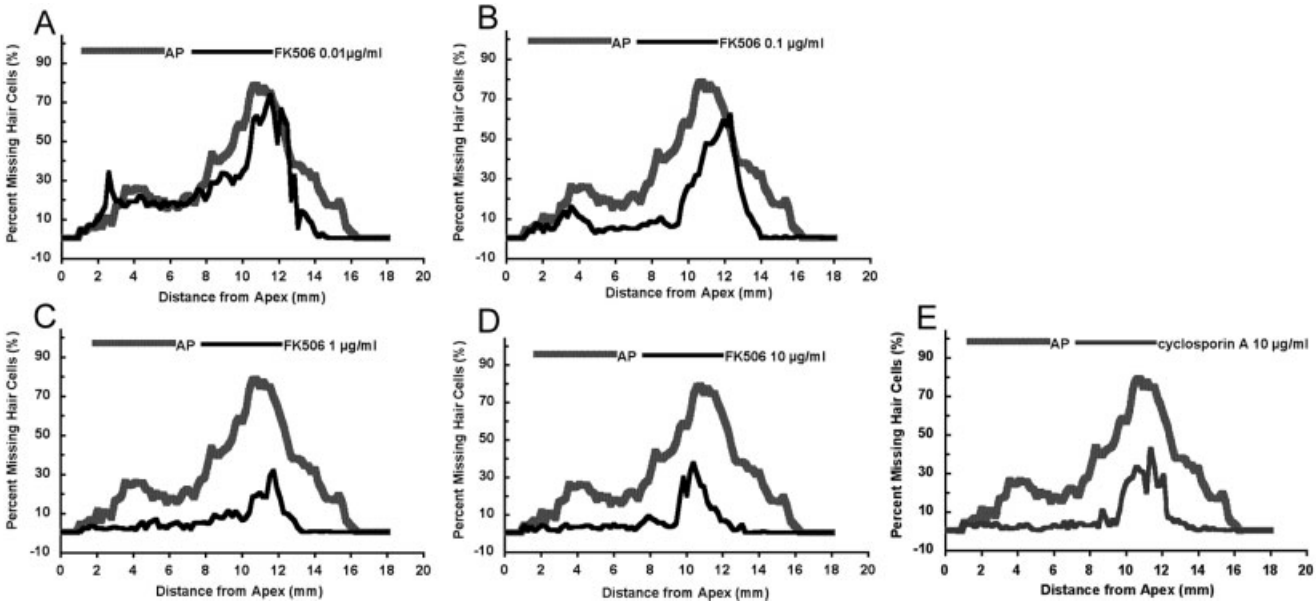


Fig. 4. Local delivery of the calcineurin inhibitor FK506 and cyclosporin A into the cochlea attenuates intense noise-induced hair loss. **A–E:** Cytocochleogram of cochleae treated with 0.01 ($n = 4$; A), 0.1 ($n = 4$; B), 1 ($n = 6$; C), or 10 µg/ml FK506 ($n = 5$; D) or 10 µg/ml cyclosporin A ($n = 5$; E) (gray line) compared to cochleae treated with AP ($n = 6$; black line). The mean percentage (± SEM) of missing OHCs in AP-treated ears was 27 ± 6%. Hair cell protection was observed to be dose-dependent with hair cell loss decreasing from 20 ± 8%, to 14 ± 5%, to 5 ± 2%, and finally to 5 ± 1%, with increasing concentrations of FK506 (from 0.01, to 0.1, to 1, to 10 µg/ml). Noise-induced hair cell loss was reduced by 1 or 10 µg/ml FK506, as well as 10 µg/ml cyclosporin A (6 ± 1%), whereas 0.01 and 0.1 µg/ml FK506 did not reach statistical efficacy.

drugs to the scala tympani of the guinea pig (Shoji et al., 2000), we observed no contralateral effect in this study. FK506 and cyclosporin A are immunosuppressants are used widely in organ transplantation for prevention of allograft rejection (Nabel, 1999). Systemic application may therefore have unwanted side effects that may be avoided by intracochlear perfusion by osmotic pump. The use of an osmotic pumps also allows relatively precise control over the timing and concentration of drugs applied to the tissues of the inner ear.

These drugs exert their effects via immunophilins, the protein receptors for these agents. Broadly speaking, there are two categories of immunophilins: the FK506 binding proteins (FKBPs), which bind FK506, and the cyclophilins, which bind cyclosporin A. Currently, the best characterized immunophilins are FKBP12 and cyclophilin A, whose complexes with FK506 and cyclosporin A, respectively, inhibit calcineurin activity. Depending on the immunophilins to which FK506 and cyclosporin A bind, FK506 and cyclosporin A have other functions in addition to calcineurin inhibition. When cyclosporin A binds to cyclophilin D, cyclosporin A can stabilize mitochondrial membranes and block mitochondrial permeability transition (MPT) (Lin and Lechleiter, 2002), and FKBP52 mediates the neurotrophic action of FK506 (Gold et al., 1999). The observation that both FK506 and cyclosporin A reduce NIHL, however, suggests that calcineurin inhibition is the important feature of the mechanism of FK506- and cyclosporin A-induced protection against noise trauma. The fact that FK506 and cyclosporin A are used clinically in humans supports the potential value of these agents to attenuate NIHL in humans.

A putative cause for noise-induced hair cell death, in addition to the initial death, caused by pure mechanical damage, is impaired cochlear metabolism and oxidative stress. Noise induces reactive oxygen species (ROS) formation (Ohinata et al., 2000); ROS scavengers and inhibitors have been implicated in protection against NIHL (Yamasoba et al., 1999; Hou et al., 2003; Ohinata et al., 2003). A preliminary experiment of a combination treatment consisting of FK506 and the ROS scavenger Trolox (vitamin E) showed no significant difference in effectiveness than with either drug alone, i.e., noise-induced ROS production and activation of calcineurin may thus be connected. Ca^{2+} influx may mediate both actions, because oxidative stress is known to both cause and be exacerbated by $[Ca^{2+}]_i$ overload (Mattson, 2000; Aarts et al., 2003; Scorrano et al., 2003). Interestingly, in vitro calcineurin inhibitors have been shown to prevent loss of neuronal cells after exposure to H_2O_2 (See and Loeffler, 2001) and β-amyloid precursor protein (Mbebi et al., 2002), each of which causes oxidative stress, calcineurin activation, and neuronal cell death. Taken together, these data implicate calcineurin activity as an important pathway in stress-induced cell death and support further the ther-

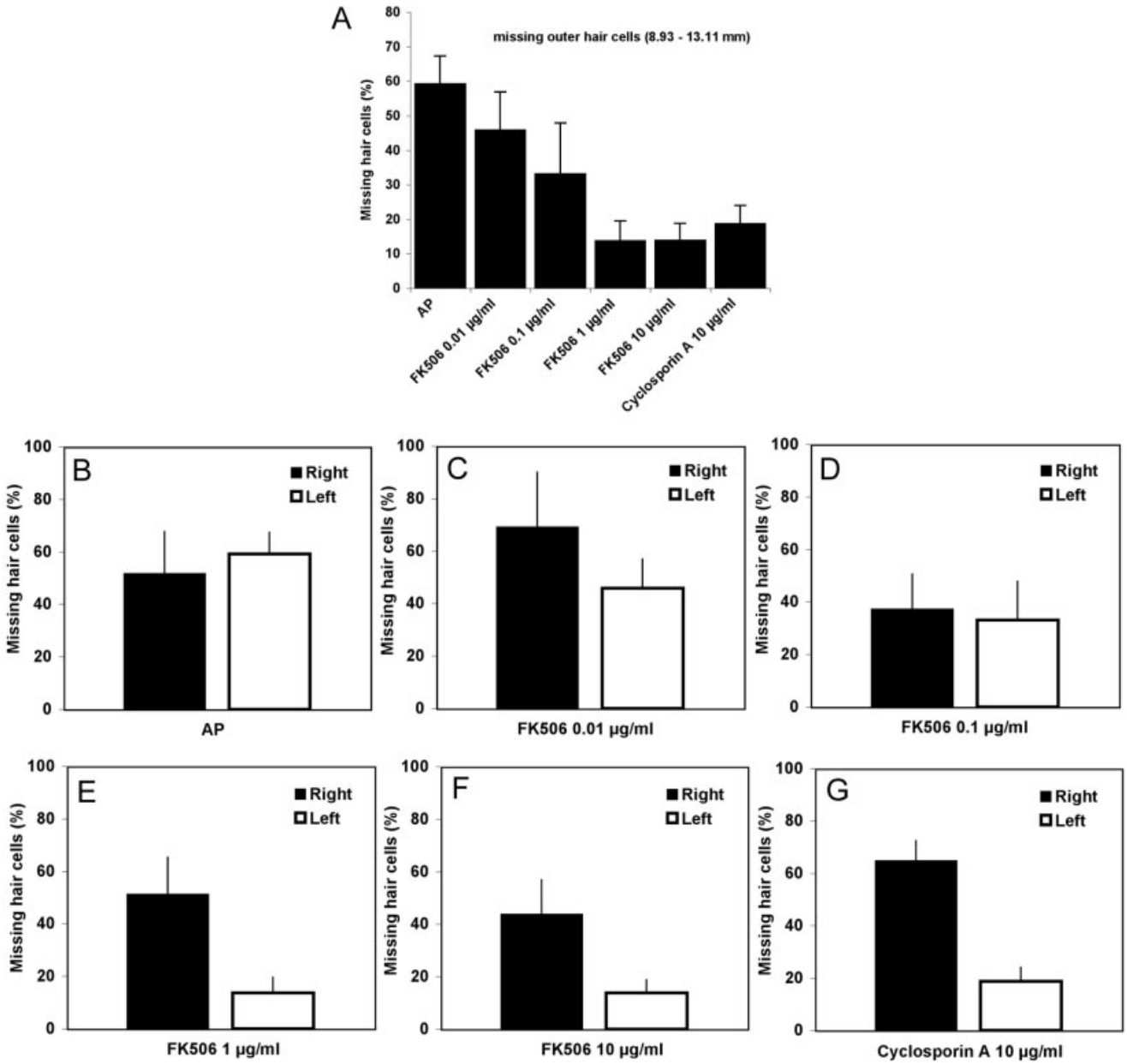


Fig. 5. FK506 and cyclosporin A reduced noise-induced hair cell loss at 8.93–13.11 mm from apex. The mean percentage (±SEM) of missing OHCs at 8.93–13.11 mm from apex was calculated for each group ($n = 6$ for AP, $n = 4$ for 0.01 μg/ml FK506, $n = 4$ for 0.1 μg/ml FK506, $n = 6$ for 1 μg/ml FK506, $n = 5$ for 10 μg/ml FK506, and $n = 5$ for 10 μg/ml cyclosporin A. **A:** FK506 at 1 and 10 μg/ml and 10 μg/ml cyclosporin A were significantly different from the AP-treated group. **B–G:** Comparisons of left cochleae perfused with AP (B), 0.01 (C), 0.1 (D), 1 (E), and 10 μg/ml FK506 (F) and 10 μg/ml cyclosporin A (G) and contralateral unperfused cochleae. Left cochleae treated with 1 and 10 μg/ml FK506 and 10 μg/ml cyclosporin A showed significantly reduced noise-induced hair cell loss as compared to that in the untreated right cochleae. Left cochleae treated with AP, 0.01, and 0.1 FK506, however, showed no significant differences compared to the untreated right cochleae.

apeutic potential of FK506 and cyclosporin A in nonneuronal and neuronal models of oxidative stress.

In summary, we have reported three interesting findings: (1) acoustic overstimulation induces calcineurin immunoreactivity at the cuticular plate region in auditory hair cells; (2) nuclei of calcineurin-positive hair cells stain with PI, which indicates dying cells; and (3) the calcineurin inhibitors FK506 and cyclosporin A reduce NIHL. These observations suggest that calcineurin is a factor in noise-induced hair cell death, and calcineurin

inhibitors may have potential clinical value to reduce NIHL.

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