Ouabain Application to the Round Window of the Gerbil Cochlea: A Model of Auditory Neuropathy and Apoptosis

R.A. SCHMIEDT,¹ H.-O. OKAMURA,^{2,3} H. LANG,¹ AND B.A. SCHULTE,^{1,2}

¹*Department of Otolaryngology and Head-Neck Surgery, Medical University of South Carolina, Charleston, SC 29425, USA*

²*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, USA*

³*Department of Otolaryngology, Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan*

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ABSTRACT

The physiological and morphological changes resulting from acute and chronic infusion of ouabain onto the intact round-window (RW) membrane were examined in the gerbil cochlea. Osmotic pumps fitted with cannulas allowed chronic (0.5–8 days) infusions of ouabain. Acute and short-term applications of ouabain (1–24 h) induced an increase in auditory-nerve compound action potential (CAP) thresholds at high frequencies with lower frequencies unaffected. The resulting threshold shifts were basically all (no response) or none (normal thresholds), with a sharp demarcation between high and low frequencies. Survival times of 2 days or greater after ouabain exposure resulted in complete auditory neuropathy with no CAP response present at any frequency. Distortion product otoacoustic emissions (DPOAEs) and the endocochlear potential (EP) were largely unaffected by the ouabain indicating normal function of the outer hair cells (OHC) and stria vascularis. One to 3 days after short-term applications, apoptosis was evident among the spiral ganglion neurons assessed both morphologically and with TdT-mediated dUTP-biotin nick end labeling (TUNEL). With 4–8 day survival times, most spiral ganglion cells were absent; however, a few cell bodies remained intact in many ganglia profiles. These surviving neurons had many of the

characteristics of type II afferents. Our working hypothesis is that the ouabain induces a spreading depression among the type I ganglion cells by blocking the Na^+, K^+ -ATPase pump. Because of the constant spike activity of these cells, the ouabain rapidly alters potassium concentrations within ($[\text{K}^+]_i$) and external to ($[\text{K}^+]_o$) the ganglion cells, thereby initiating an apoptotic cascade.

Keywords: apoptosis, auditory nerve, endocochlear potential, gerbil hearing, osmotic pump, ouabain spreading depression

INTRODUCTION

Ouabain is a well-known cardiac glycoside that specifically binds to Na^+, K^+ -ATPase and blocks its activity. The Na^+, K^+ -ATPase pump plays a fundamental role in cellular ion homeostasis (Alberts et al. 1983). The enzyme is a heterodimer composed of one α and one β subunit, with the α subunit being the site of attachment for ($[\text{K}^+]_o$) and ouabain (Lingrel and Kuntzweiler 1994; Lingrel et al. 1998). Ouabain has been used as a tool in many previous investigations of cochlear ion homeostasis (Konishi and Mendelsohn 1970; Konishi and Salt 1980; Marcus et al. 1981b), cochlear potentials (Kuijpers et al. 1967; Konishi and Mendelsohn 1970; Kuijpers and Bonting 1970; Sellick and Johnstone 1974; Boshier 1980), and function of the stria vascularis (Marcus et al. 1978, 1981b; Wangemann et al. 1995). It has been shown in most species to be quite effective in blocking the transport of K^+ into

Correspondence to: Dr. R.A. Schmiedt • MUSC/ENT Research • P.O. Box 250150 • Charleston, SC 29425. • Telephone: (843) 792-7977; fax: (843) 792-7736; email: schmiera@musc.edu

scala media and reducing the endochlear potential (EP) when applied via a perilymphatic route (Kuijpers et al. 1967; Kuijpers and Bonting 1970; Kusakari et al. 1978; Bosher 1980; Klis and Rebillard 1995).

This study evolved from our efforts to understand the functional consequences of impeding the activity of the stria vascularis, a structure which has been implicated as a primary site of age-related pathology leading to hearing loss in quiet-reared gerbils (Schulte and Schmiedt 1992; Gratton et al. 1995; Schmiedt 1996). One aspect of our studies is the development of a model of age-related hearing loss in a young animal. Furosemide has been used successfully as an agent to block the generators of the EP when applied chronically to the round window (RW) via an osmotic pump and cannula (Schmiedt 1997; Schmiedt et al. 2000). It was thus expected that ouabain similarly applied to the RW might be even more effective than furosemide in inducing long-term injury of the stria. Given the previous studies in other species, our original hypothesis was that ouabain would target the marginal cells and possibly some of the fibrocytes located within the lateral wall which stain positively for Na^+, K^+ -AT-Pase (Schulte and Adams 1989; McGuirt and Schulte 1994; Gratton et al. 1995, 1997).

Surprisingly, the results in the gerbil revealed that the spiral ligament and stria vascularis were not permanently affected by the acute or chronic application of ouabain to the RW. Indeed, the EP remained relatively stable in these ears, as did outer hair cell (OHC) function as monitored with DPOAEs. Instead, ouabain treatment promoted a partial to complete and permanent loss of auditory-nerve function. Further investigation at the light microscopic (LM) level revealed that exposure to ouabain selectively destroyed most of the spiral ganglion neurons, leaving only a few remaining. Thus, the application of ouabain to the RW of the gerbil cochlea produces a model of auditory-nerve neuropathy, while leaving the stria and hair cell systems relatively intact.

METHODS

Animals

Thirty-seven Mongolian gerbils (*Meriones unguiculatus*) of both genders and with healthy external ears were used in these experiments. Ages ranged from 4 to 7 months. Twenty-one animals were implanted with pumps with cannulas leading to the right ear; 16 animals were used in the acute experiments. Left ears were always used as controls. Animals were born and reared in an acoustically controlled colony where median sound pressure levels (SPL) were seldom greater than 40 dBA. The animal facilities have full AAALAC accreditation, and all experimental proce-

dures were approved by the local IACUC and met NIH guidelines for animal care.

Surgical procedures

Surgery and the procedures for the recording of the compound action potential (CAP) response and EP have been described previously (Schmiedt and Zwislocki 1977; Schmiedt 1996; Schmiedt et al. 1996). Briefly, the animal was anesthetized with sodium pentobarbital (50 mg/kg) and fitted to a head holder located in a sound- and vibration-isolated booth. The booth was heated to maintain the cochlea at or near body temperature. Supplemental doses of anesthesia were given as needed. The core temperature of the animal was controlled by a closed-loop DC heating pad. The pinna and surrounding glands were removed and the bulla opened widely.

Acute applications of ouabain to the RW used about 100 μL of 1 mM ouabain (diluted with normal saline) to fill the RW niche. CAP thresholds were monitored during this procedure with a Ag-AgCl wire electrode resting on the surface of the bony niche. When a recovery period was desired, the RW niche was accessed using sterile procedures via a lateral incision ventral to the pinna. The ouabain was washed off after 1–2 h, depending on the acute CAP threshold shift desired. The bulla was then cemented closed, the incision sutured, and the animal allowed to recover for between 12 h and 1 week. To minimize cochlear trauma, no measures of EP were made during the actual application of ouabain to the RW for acute applications associated with a recovery period. There were no recovery periods in those acute experiments where EP was monitored continuously during ouabain application (see Figs. 4 and 5).

The survival surgery for pump implantation was done under sterile conditions. Antibiotics were not used, and none of the animals showed signs of any past or ongoing infection. Gerbils were anesthetized with sodium pentobarbital (50 mg/kg) and were given atropine (0.2 mg/kg) to reduce secretions. Alzet® mini-osmotic pumps (model 2004, Durect, Cupertino, CA) were used in all the experiments. These pumps have a fill volume of about 200 μL and a mean pumping rate of 0.25 $\mu\text{L}/\text{h}$ at 37°C. Under these conditions they will last about 28 days. Cannulas were made up of surgical-grade silicon tubing. Pumps and cannulas were filled aseptically and allowed to equilibrate at 37°C for 48 h before implantation. Pumps were filled with 1 mM ouabain (diluted with sterile normal saline) and were left implanted for 12 and 24 h (short-term), or 2, 4, and 8 days (chronic). In some gerbils, terminal physiological measures were done with the pump in place; in others, the pump was removed and the animal allowed to recover for 1–4

weeks before terminal physiology was obtained. The pump was placed subdermally behind the scapulae and the cannula led to the bulla. The end of the cannula was threaded through holes in the bulla and placed inside the RW niche (Chamberlain 1977), then fixed in place at the surface of the bulla with dental cement. The bulla was fully closed with the cement. Incisions were closed with sutures and the animal allowed to recover in its cage on a heating pad. Post-surgical discomfort was treated with buprenorphine, but most animals showed no signs of discomfort.

Electrophysiological procedures

The CAP electrode was placed on the bony rim of the RW niche, and the acoustic assembly comprising a probe-tube microphone (Bruel & Kjaer 4134) and driver (Beyer DT-48) was sealed to the bony ear canal with closed-cell foam. In implanted ears, the cannula was left in place during the CAP and EP recordings. CAP thresholds were obtained at half-octave frequencies from 0.5 to 16 kHz and at 20 kHz. The tone pips were generated in the frequency domain by TDT® (Tucker Davis Technologies, Gainesville, FL) equipment and software, and the spectrum was normalized to the average ear canal SPL obtained from 30 gerbils. The tone pips were 1.8 ms in total duration with a $\cos^2$ rise/fall time of 0.55 ms. The same tone pips were used to obtain CAP I/O functions at 1, 2, 4, 8, and 16 kHz. The I/O functions were incremented from 20 to 90 dB SPL in 10 dB steps.

Endocochlear potentials were recorded in cochlear turns 1, 2, and 3 corresponding to respective best frequencies of about 16, 2.2, and 1 kHz according to the single-fiber map of Müller (1996). EP microelectrodes were filled with 0.2 M KCl and were between 30 and 50 M Ω impedance. EP in each turn was always measured as the voltage difference between scala media and a pool of isotonic saline on the neck muscles.

DPOAEs were measured with an ARIEL board and CUBeDISP™ software using the B&K 4134 microphone, probe tube, and frequency equalizer. The intensity levels of both primaries were fixed at 50 dB SPL. Other primary levels were used, mostly $L_1 = 50$, $L_2 = 40$ dB SPL, but the results did not differ substantially from those obtained with $L_1 = L_2 = 50$ dB SPL and are not shown in this report. Primary tones were swept from $f_2 = 20.0$ to 0.5 kHz with an f_2/f_1 ratio of 1.2 and a resolution of 10 points per octave. For clarity in the figures presented here, every other point was omitted for a resolution of 5 points per octave. Identical procedures were always done in both the left (control) and right ears of each animal. Additional methodology for obtaining the DPOAEs can be found in Boettcher and Schmiedt (1995).

Complete physiological evaluations, including CAP thresholds and I/O functions, DPOAEs, and EPs were obtained in all the ears examined in this study.

Morphological procedures

For histochemical studies, anesthetized animals were exsanguinated by transcardial perfusion with 10 mL of 0.9% saline solution containing 0.1% sodium nitrite followed by 50 mL of 10% formalin in 0.9% saline containing 0.5% zinc dichromate (pH 5.0). The temporal bones were then resected and fixed for 1 h by immersion. The ears were decalcified in EDTA, dehydrated, embedded in paraffin, and sectioned at 6 μ m. Deparaffinized and rehydrated sections were incubated overnight at 4°C with a 1:1000 dilution of polyclonal rabbit anti-calretinin antibody (Zymed Laboratories, South San Francisco, CA), flooded with biotinylated secondary antibody, incubated in Vectastain® ABC reagent (Vector Laboratories, Burlingame, CA), and developed by incubation in 3,3'-diaminobenzidine (DAB) in H₂O₂ substrate medium. Further methodological details can be found in Schulte and Adams 1989 and Schulte and Schmiedt 1992. Sections used for the TUNEL procedure were deparaffinized, rehydrated, and immersed in TdT (terminal deoxynucleotidyl transferase, Boehringer Mannheim, Indianapolis, IN) solution for 40 min at room temperature. After stopping the reaction, the sections were incubated with peroxidase conjugated anti-digoxigenin and reacted with DAB-H₂O₂ substrate medium to visualize binding sites.

Tissue preparation for embedment in plastic was similar to that described above but a freshly prepared mixture of 4% paraformaldehyde–2% glutaraldehyde was substituted for the zinc-formalin fixative. The inner ears were then immersed in fixative overnight at 4°C, decalcified with EDTA, and postfixed with a 1% OsO₄–1.5% K₄Fe(CN)₆ solution for 2 h in darkness. Specimens were dehydrated, embedded in Epon LX112 resin, sectioned to a thickness of about 1 μ m, and stained with toluidine blue. A more complete description of these methods for processing tissue for ultrastructural evaluation can be found in previous work (Spicer et al. 1999).

RESULTS

Physiology

CAP threshold data and EP values were obtained from six gerbils with short-term (12–24 h) pumps. Data from one of these gerbils are shown in Figure 1A. The physiological data were obtained 12 h after the implant, so the total volume of 1 mM ouabain delivered to the RW niche of the right cochlea was

FIG. 1(A). CAP thresholds obtained after 12 h of ouabain treatment via an implanted pump with no recovery. Filled circles show data from the right ear, which in this study was always the ear treated with ouabain. The open squares plot data from the left, control ear. The left ear shows normal sensitivity with regard to CAP thresholds for the gerbil. There is a sharp demarcation in the thresholds obtained from the ouabain-treated ear, changing from normal to no response (indicated by caret) in the frequency interval between 2.8 and 5.6 kHz (90 dB SPL was the maximum presentation level allowed). The downward arrow indicates the frequency at which CAP input/output (I/O) functions shown in **B** were obtained. Note that the EP measures in the base (turn 1 at 16 kHz) and apex (turn 3 at 1 kHz) were unaffected by the ouabain treatment. **(B)** CAP input/output (I/O) functions obtained from the same ear at 2 kHz as indicated by the arrow in **A**. Note that even though the CAP thresholds are normal, CAP amplitudes at high SPLs are dramatically reduced when compared with the control ear. Thus, in the early stages of ouabain toxicity, the neural response at suprathreshold levels can be reduced without a subsequent elevation of thresholds or decrease in EP.

about 3 μ L. The CAP thresholds of the control (left) ear are within the normal profile for young gerbils as are the EP measures recorded in the first turn (T1, 16 kHz) and in the third turn (T3, 1 kHz) (Schulte and Schmiedt 1992; Schmiedt 1993, 1996; Hellstrom and Schmiedt 1996). There were no differences in EP values measured in the pump and control ears in this animal. However, CAP thresholds were unmeasurable in the pump ear at 5.6 kHz and above, but were normal and matched those of the control ear at 2.8 kHz and below. This sharp demarcation between normal and highly elevated CAP thresholds was always seen with short-term pump applications (12–24 h) and no recovery period or with acute administration of ouabain (1–2 h) with a 12 h recovery period.

FIG. 2(A). CAP thresholds from a gerbil treated for 8 days with ouabain and allowed to recover for 4 weeks. The right, pump ear shows no CAP response at 90 dB SPL (caret) at any frequency. The thresholds of the left, control ear are normal. Again EP measures in the base and apex were similar for both ears, indicating that the stria was little affected by this route of ouabain treatment. **(B)** DPOAEs obtained with constant-level ($L_1 = L_2 = 50$ dB SPL) primaries from the ouabain-treated (right) and control (left) ears of the same gerbil shown in **A**. Dotted line (noise) indicates the real-time noise floor of the system. Note that there is little difference between the ears. The DPOAEs remained robust under all conditions of ouabain treatment. Given that DPOAEs obtained with low-level primaries are a good indicator of outer hair cell function, the outer hair cell system, like the stria, seems to be little affected by the application of ouabain to the RW.

CAP I/O functions were examined in both ears of animals with this threshold profile to test supra-threshold neural responses at low frequencies where thresholds were normal (Fig. 1A, arrow). Figure 1B demonstrates that even though the thresholds were similar for both ears, the 2 kHz CAP response in the pump ear was clearly degraded at suprathreshold intensities. In all the animals with this threshold profile, CAP I/O functions taken at 1 and 2 kHz in the normal threshold region showed similar reductions in their maximum amplitudes obtained at high SPLs compared with the control ear. Thus, in the early stages of ouabain toxicity neural thresholds could be unaffected, whereas high-level CAP responses were dramatically reduced.

In the 13 gerbils which were allowed to recover for 2 days to 4 weeks, either after acute application of ouabain or after chronic pump application for periods of

FIG. 3. Mean EP and standard deviations from 13 ouabain-treated gerbils combining data from both the acute group and the pump-implanted groups. In these 13 gerbils, EP was measured in all three turns of both the ouabain-treated and control cochleas. There were no significant differences between the ouabain and control groups in any of the turns using either the standard T test or one-way ANOVA. There were also no significant differences between control and ouabain-treated data when the acute or pump-implanted animals were treated separately.

12 hours to 8 days, the findings were similar: neural responses were always absent as measured by CAP procedures. This condition is typified by the data shown in Figure 2A. Here the gerbil was treated with ouabain via a pump for 8 days, then allowed to recover for 4 weeks. There was no CAP response detected in the treated ear, whereas the control ear was found to be normal. Note that the EP measures recorded in both ears were similar, as in Figure 1A, demonstrating that ouabain had no discernable permanent affect on strial function. EP measures were normal or near normal for all cochleas treated with ouabain delivered via a pump or allowed to recover from an acute application.

To judge whether OHC function was compromised by 1 mM ouabain treatment, DPOAEs were measured in both ears of all animals ($n = 37$). In all cases, 1 mM ouabain did not significantly alter DPOAE magnitudes (Fig. 2B). Here, DPOAEs derived from constant-level primaries are plotted from the same gerbil as in Figure 2A. There was no significant loss of DPOAE magnitudes in the treated ear. Thus, the presence of robust DPOAEs to low-level primaries indicates that ouabain applied to the RW of

FIG. 4(A). Basal turn EP as a function of time after acute application of 100 μ L of 1 mM ouabain to the RW niche. EP was recorded continuously with a micropipette placed through the RW. Arrow indicates the time of application of ouabain which was not washed out in this experiment. **(B)** CAP thresholds obtained before (Pre) and 180 min after (Post) the application of ouabain. Note the total loss of the CAP response to high-frequency tone pips. Data from other animals allowed to recover from this type of exposure indicate that the CAP response would be totally absent after 12 h and not recover. **(C)** DPOAEs obtained with constant level ($L_1 = L_2 = 50$ dB SPL) before (Pre) and 180 min after (Post) application. The ouabain had little effect on the DPOAEs. Thus, the EP can be decreased by applications of ouabain at the RW of the gerbil and, unlike the CAP response, can recover from this concentration level.

the gerbil cochlea acts most specifically on the neural response, leaving OHC function largely intact.

Ouabain applied at 1 mM had little affect on the EP, as shown by the mean data from 13 gerbils in Figure 3. These gerbils had a complete set of EP data obtained from all three turns of both cochleas. The means encompass both acute experiments with a recovery period and pump-implanted animals. Even when grouped according to specific protocol, e.g., acute, pump with recovery, pump without recovery, no significant differences were found between the ouabain-treated and control cochleas with regard to EP values in any of the turns.

A closer examination of how acute ouabain exposure affects cochlear physiology in the gerbil is provided in Figures 4 and 5. A time series of EP measures was examined in four ears of two gerbils. Panel A of Figure 4 demonstrates the time course of the EP after 100 μ L of 1 mM ouabain was applied to the RW niche

FIG. 5(A). A time series similar to that in Figure 4 obtained from another animal with 100 μ L of 10 mM ouabain applied at times indicated by arrows. Arrows subsequent to the first are times when the ouabain was washed out with saline and then reapplied at the original concentration. The last arrow indicates a washout with no additional application of ouabain. The EP clearly is decreased by the ouabain at this higher concentration. **(B)** The CAP response obtained before (Pre) and 360 min after (Post) the time series shown in **A**. The CAP was totally absent by 3 h after the initial exposure. Pre-exposure thresholds were normal. **(C)** DPOAEs obtained with 50 dB primaries before (Pre) and 360 min after (Post) the initial application. While there is a consistent decrease in the post-DPOAE levels across frequency, they are still far above the noise level. Note that when the post-DPOAEs were obtained the EP had dropped to about 20 mV (**A**).

with no washout. The arrow indicates the time of application. The EP does drop significantly over the first 60 min, but recovers to pre-exposure values within about 120 min, then remains stable until the end of the recording at 180 min. In contrast, as shown in Figure 4B, the CAP response after 180 min is absent for frequencies above 4 kHz, whereas thresholds remain normal at lower frequencies. Finally, the DPOAE magnitudes obtained with low-level primaries shown in panel C are unchanged from pre-exposure values after 180 min, again demonstrating that OHC function remains robust after acute ouabain exposure.

A similar time series obtained with a higher concentration of ouabain (10 mM) is shown in Figure 5A. Here the ouabain was repeatedly applied to the RW in increments of 100 μ L, left for a period of time, then washed out with saline and reapplied (arrows). It is clear that high doses of ouabain applied via the RW in gerbil can affect the EP, a result previously found in chinchilla (Rybak et al. 1984). By the end of this time

FIG. 6. Ouabain-induced spiral ganglion neuropathy. An antibody against calretinin was used as a specific marker for afferent neurons. Shown are ganglion-cell profiles in the basal turn of a control ear (**A**) and ouabain-treated ears with pump durations of 12 h (**B**) and 8 days (**C**). Note that even at 12 h, there was some disruption of ganglion cells. There was a total loss of ganglion cells in this profile after 8 days of treatment, although a few residual ganglion cells were frequently present in many sections. Scale bar = 50 μ m.

series (360 min), the corresponding CAP response was absent at all frequencies (panel B). In contrast, as shown in panel C, the DPOAE magnitudes at 360 min were somewhat diminished from pre-exposure values, probably a result of the small residual EP (~20 mV), an extreme condition which would be expected to reduce the effective gain of the cochlear amplifier.

In the acute exposures, a change was sometimes noticed in the CAP waveform (a broadening of the response) before any shift in the CAP threshold occurred during the ouabain application. If the ouabain was washed out at this point and the animal was allowed to recover for a week, either no loss of CAP thresholds or a complete loss of neural potentials was observed. On the other hand, if the ouabain was not washed off until the CAP thresholds associated with the basal turn began to rise by 10 dB or more, the neural response was always totally absent after one week of recovery. Thus, there seems to be a critical point for this all-or-none neuropathy.

Morphology and histochemistry

Representative midmodiolar sections through the spiral ganglion of the basal turns (T1) of three ears

FIG. 7. Neuronal death is mediated by apoptosis. **(A)** Plastic section from basal turn of an ear exposed to ouabain via a pump for 24 h with no recovery period. Classic signs of apoptosis (white arrows) are present in the nuclei of several ganglion cells. **(B)** Same region from the opposite ear showing normal ganglion cells in a control cochlea. **(C)** The TUNEL procedure shows many nuclei intensely labeled in the ear treated with ouabain for 12 h, whereas no reactivity is present in the opposite control ear **(D)**. Scale bar for **A** and **B** = 10 μm ; scale bar for **C** and **D** = 50 μm .

immunostained with anti-calretinin, a specific marker for afferent neurons and IHCs (Dechesne et al. 1993; Pack and Slepecky 1995), are depicted in Figure 6. Panel A shows a normal ganglion cell profile from an unexposed ear, and panels B and C show profiles of ganglia from ears exposed to ouabain for 12 h and 8 days, respectively, with no recovery period. Ganglion cell soma are disrupted but are still present at 12 h. By 8 days of drug treatment, no staining is evident in neurons or their processes, indicating an almost complete loss of ganglion cells. This pattern of loss was generally representative for all turns in ears with no CAP response, although a few relatively normal-appearing ganglion cells were seen in many ganglia profiles. Despite the apparent loss of afferent innervation, inner hair cells continued to stain strongly with antibody to calretinin and showed no discernable histopathologic changes (Fig. 6).

Morphological examination of plastic sections from well-preserved control and ouabain-treated ears provided further information. Many ganglion cells in ears with short-term treatments (24 h or less) with no recovery period showed classic signs of apoptosis having condensed, pyknotic, and crenelated nuclei (Fig. 7A). Control ears exhibited normal morphology with no signs of nuclear fragmentation (Fig. 7B). The presence of apoptotic cell death was confirmed by TUNEL staining as shown in Figures 7C and 7D. Numerous nuclei intensely reactive with the TUNEL method were detectable as early as 12 h after exposure to ouabain (Fig. 7C), whereas

sections from control ears were consistently unstained (Fig. 7D).

Semithin plastic sections through the basal turn of a control ear showing normal ganglion cell morphology are shown in Figures 8A and 8B. Type I and a small number of type II afferent neurons are normally present in the ganglia and can be distinguished from one another in semithin sections. Figure 8C illustrates a similar region in an ear treated with a pump for 12 h and then allowed to recover for 2 weeks. The few surviving ganglion cells in this profile have morphological characteristics similar to those of the type II afferents seen in Figure 8B.

DISCUSSION

Effects of RW-applied ouabain on cochlear function in gerbil

The main result of this study is that ouabain, when applied to the RW of the gerbil cochlea, can selectively and permanently destroy spiral ganglion neurons while leaving the hair cell and strial systems relatively intact. These conclusions stem from experiments where 1 mM ouabain was applied acutely for a minimum of 1 h and the animal was allowed at least 12 h to recover, or when it was applied chronically via a pump for at least 12 h with or without a recovery period. Even though there is total nerve deafness in these cochleas, DPOAEs and the EP remain robust, indicating that the OHC and strial systems, respec-

FIG. 8. Ouabain-resistant spiral ganglion neurons in an animal treated for 12 h and then allowed to recover for 2 weeks. (A, B) Semithin sections taken from the basal turn of the control ear. (B) Boxed area from (A) at a higher magnification illustrates morphology of type I myelinated and type II unmyelinated ganglion cells. (C) Two surviving ganglion cells in the basal turn of the ouabain-treated ear. These residual cells, which strongly resemble type II cells, were seen in all regions of the spiral ganglia. Scale bars for A and C = 50 μm ; scale bar for panel B = 20 μm .

tively, are functionally intact. The physiological data are supported by the histopathological results showing clear evidence of apoptosis in the early stages of toxicity leading to an almost total loss of ganglion cells after about two days. Thus, the demise of the ganglion cells is quite rapid and follows a course indicative of a spreading depression.

Comparison to previous studies

Most previous studies on the effects of ouabain on the cochlea have employed direct perilymphatic perfusion of the drug on an acute basis with no recovery period. This treatment resulted in large and rapid alterations of the cochlear microphonic (Kuijpers and Bonting 1970), the EP (Konishi and Mendelsohn 1970; Bosher 1980; Rybak et al. 1984), and the Na^+ and K^+ concentrations in perilymph and endolymph (Juhn and Pearce 1977; Konishi and Hamrick 1978; Konishi et al. 1978; Kusakari et al. 1978). Vanadate, a less specific inhibitor of $\text{Na},\text{K}\text{-ATPase}$, was an exception to the above studies. When perilymphatically perfused, vanadate markedly decreased the cochlear microphonic but had little effect on the EP (Marcus

et al. 1981a). Marcus et al. concluded that vanadate mainly affected hair cells and not stria function.

We did not routinely monitor the EP during acute exposures. It was felt that the necessary recording procedures were too invasive and could introduce trauma and pathologies not related to ouabain toxicity. However, to explore the question of the acute effects of ouabain on cochlear potentials in the gerbil, the EP was monitored in three animals during the acute application of ouabain. The results in Figure 4 show that a single exposure of ouabain at a concentration of 1 mM decreased the EP, but only on a short-term basis (1 h). This short-term clearance of ouabain may be an indication of the diffusion kinetics present in the perilymph after a RW application of the drug (Salt and DeMott 1997, 1998, 1999). At a concentration of 10 mM (Fig. 5), a significant decline of the EP was apparent, a trend similar to that seen at a 1 mM concentration in chinchilla (Rybak et al. 1984). Thus, the EP in gerbil seems more resistant to ouabain applied via the RW than in other species. Finally, the data shown in Figure 5 demonstrated that DPOAE magnitudes taken with low-level primaries can remain moderately strong even when associated with an EP as low as 20 mV. This independence between DPOAEs and EP is probably a manifestation of the ability of cochlear hair cells to adapt to EP changes, as first reported by Mills et al. (1993).

In the morphological study of Hamada and Kimura (1999), ouabain was applied in guinea pig in a fashion similar to that employed here: directly to the RW with a recovery period as well as via an osmotic pump. The pump effluent, however, was allowed to infiltrate the animal systemically; i.e., a cannula to the RW was not used. Their results clearly demonstrated that the marginal and intermediate cells of the stria, fibrocytes in the spiral ligament, and sensory cells were most affected by the ouabain. Spiral ganglion neurons exhibited some shrinkage compared with normal neurons but did not degenerate completely as found here in the gerbil.

Several factors may explain why Hamada and Kimura's (1999) results differ from those reported here. First, they applied small doses of ouabain (5 μL of 2 mM twice a day) to the RW for 2–3 days and then sacrificed the animals 2–7 days after the last dose. Perhaps this pulsed low dose was insufficient to initiate a spreading depression in the nerve (see below). Second, there are differences in the cochlear anatomy between the gerbil and guinea pig. In the gerbil the RW niche is separated from the auditory nerve and the spiral modiolar artery by an extremely thin wall of bone (Sokolich et al. 1976; Chamberlain 1977). In contrast, the auditory nerve in the guinea pig is much less exposed and lies deeply in the temporal bone with considerably greater separation from

the RW niche (Axelsson 1968; Asarch et al. 1975; Nadol 1988). Thus, it is likely that ouabain has much easier access to the nerve and its associated blood vessels in the gerbil compared with the guinea pig, resulting in higher concentrations within the modiolus.

A third reason for differences between our study and previous studies may be the well-known fact that ouabain sensitivity is species and cell type related. For example, neurons can differ in their sensitivity when compared with skeletal muscle (Sweadner 1985). Such species and tissue differences are related to the presence of various isoforms of Na^+, K^+ -ATPase. The enzyme is a heterodimer composed of two subunits, α and β , of which α is most important with regard to the binding of $[\text{K}^+]_o$ and ouabain (Lingrel and Kuntzweiler 1994; Lingrel et al. 1998). There are at least three isoforms of the α subunit: $\alpha 1$, $\alpha 2$, and $\alpha 3$. The $\alpha 3$ subunit is most commonly found in neuronal tissue, including the gerbil spiral ganglion neurons (McGuirt and Schulte 1994), along with the $\beta 1$ isoform. Interestingly, the $\alpha 3$ subunit also is the most sensitive to ouabain (Hara et al. 1988). Site-directed mutagenesis has shown that substitution of a single amino acid in the α subunit isoform can induce a large difference in the sensitivity of Na^+, K^+ -ATPase to ouabain (Lingrel et al. 1998). Thus, it is possible that the auditory neurons in gerbil are more sensitive to ouabain than those in guinea pig or chinchilla because of species differences in the structure of the $\alpha 3$ subunit.

The concept of spreading depression and the survival of type II afferents

Inhibition of Na^+, K^+ -ATPase prevents neurons from re-establishing their normal Na^+ and K^+ gradients after an action potential. As a result, each spike incrementally depolarizes the axon until the membrane potential approaches zero (Alberts et al. 1983). Thus, it is understandable why the survival of highly active, spiking neurons—like those in the auditory nerve—might be exquisitely sensitive to ouabain. Indeed, many of the mechanisms that are known to induce apoptosis in nerve and muscle cells involve K^+ homeostasis. For example, apoptosis can result by lowering $[\text{K}^+]_i$ (Perez et al. 2000; Krick et al. 2001), by maintaining cell depolarization over an extended time (Bortner et al. 2001), or by other mechanisms that are intimately tied to an efflux of K^+ from the cell (McLaughlin et al. 2001).

Ouabain has been used to induce the phenomenon known as spreading depression wherein the death of one neuron triggers the demise of its neighbors (Grafstein 1956; Menna et al. 2000). This often leads to a positive feedback cycle where extra-

cellular $[\text{K}^+]_o$ rises to toxic levels because of the inhibition of the Na^+, K^+ -ATPase both in nerve fibers and in surrounding glial cells (Balestrino et al. 1999). The high $[\text{K}^+]_o$ in turn affects surrounding neurons by depolarizing them and making them even more susceptible to ouabain toxicity. Xiong and Stringer (2000) recently showed that the ouabain-induced spreading depression in the dentate gyrus of the hippocampus is caused mostly by the neuronal inability to clear $[\text{K}^+]_o$ and not so much from the inhibition of the glial clearing of $[\text{K}^+]_o$.

Because their underlying causes often rely on positive feedback, spreading depressions tend to be all-or-none events. The all-or-none characteristics of the ouabain-induced neuropathy reported here support the hypothesis that a spreading depression phenomenon is involved. We have tried to titrate the steady-state (i.e., recovered) neural pathology resulting from ouabain application, the goal being a stable audibility curve with a sharp frequency boundary between normal and absent responses like those shown in Figures 1 and 4. Our attempts were not successful.

The presence of a small population of relatively normal-appearing ganglion cells after ouabain treatment suggests that their survival is not as dependent on Na^+, K^+ -ATPase pump activity. If these surviving neurons are indeed type II afferents from OHCs, our results suggest that they do not carry action potentials, or they have an extremely low spike activity (Robertson et al. 1999).

Ouabain toxicity and apoptosis

The data clearly show that ouabain treatment induces apoptosis in spiral ganglion neurons (see Fig. 7). Previous studies have shown that apoptosis can be initiated in neurons by oxidative stress (Huang et al. 2000), nitric oxide (Zdanski et al. 1994; Wu et al. 1997), aging (Zheng et al. 1998), and treatment with cisplatin (Alam et al. 2000). Apoptosis can also be initiated in neural tissue by spreading depression (Iijima 1998; Stoll et al. 1998) and by lowering the concentration of intracellular K^+ (Isaev et al. 2000). With ouabain poisoning, auditory neurons with their high spike activity would quickly lose intracellular K^+ , so the latter is a likely pathway to initiate apoptosis.

Future areas of study

The ouabain model described here could be important for the study of spreading depression in auditory fibers, apoptosis induced by ionic imbalance, IHCs under conditions of isolation from their afferent fibers, and type II ganglion cells and their connections. Our morphological studies are also incomplete in

terms of detailed cell counts and the effects of ouabain on efferent fibers. It is entirely possible that by titrating the dose and recovery time, in conjunction with blockers of apoptosis, one could obtain a range of pathologies from a cochlea devoid of neural elements, to one that had just type II fibers, to one with a scattered loss of neural elements. The latter model would be especially appropriate for aging studies where diffuse ganglion cell loss is often present.

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