Effect of selective damage to cochlear outer hair cells on alternating current induced otoacoustic emissions

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The relationship between alternating current (AC)-induced otoacoustic emissions (OAEs) and the cochlear outer hair cells (OHCs) was examined. Injection of an AC into the scala media of the guinea pig cochlea, together with simultaneous stimulation by a pure tone, induced sound emissions in the ear canal at the frequencies of the AC and intermodulation distortion products. Amplitude of these emissions changed depending on the frequency of the injected AC or stimulating tone. The amplitude and its frequency dependence were decreased by selective damage to OHCs by ototoxic agents, and the decrease depended on the extent of the OHC damage, especially at the AC injection site. It is suggested that OHC acts as an electromechanical transducer in the cochlea and is responsible for the generation of the AC-induced OAEs. A nonlinearity to produce the intermodulation distortion was more susceptible to the OHC damage than the electromechanical transducing process.

Keywords: AC-induced otoacoustic emissions, Outer hair cells, Ototoxic agents, Histochemical dyeing of hair cells, Guinea pig

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1. INTRODUCTION

Many studies on otoacoustic emissions (OAEs) have been reported since the first report by Kemp.^{1,2)} At present, although it is generally accepted that the source of OAE generation exists in the cochlea, the exact mechanism and the physiological meaning of OAEs are not yet clear. One of the reasons may be that, except for distortion-product OAEs, the incidence of OAEs is much lower in animals than in humans.^{1,3)}

Hubbard and Mountain⁴⁾ have described that OAEs are induced by an alternating current (AC) injected into the scala media of the gerbil cochlea. We also observed a high incidence of the same AC-

induced OAEs in guinea pigs and rats. We have shown already that the AC-induced OAEs are originated in the cochlea since normal metabolic activity in the cochlea is essential and the emissions are regulated by the central nervous system through the olivocochlear bundle.^{5,6)} It has been well established that isolated outer hair cells (OHCs) show a motile response to an electrical stimulation.⁷⁻¹³⁾ These facts suggest that, *in vivo*, OHCs can vibrate actively in response to electrical stimulation and generate AC-induced OAEs.

In the present study, we applied ototoxic agents to the guinea pig in order to damage OHCs and examined the effects of the OHC damage on the AC-induced OAEs. The hair cells were dyed histochemically, and the morphological and functional changes of OHCs were examined.

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2. MATERIALS AND METHODS

2.1 Selective Damage to OHCs

Ninety-five healthy guinea pigs, weighing between 250 and 500 g and showing normal Preyer's reflex, were used for the experiments. OHCs were damaged by applications of nitrogen mustard-N-oxide hydrochloride (Nitromin, Yoshitomi Pharmaceutical Industries, Ltd.) and kanamycin sulfate (kanamycin, Meiji Seika Kaisha Ltd.). In 36 guinea pigs, a single shot of Nitromin (10–40 mg/kg) was applied intraperitoneally and they were used for the experiments 2–9 days after the application. In 8, the experiments were performed after intramuscular applications of kanamycin (400 mg/kg/day) for 13–20 days. As a control, the other 51 guinea pigs were not applied any ototoxic agents.

2.2 Measurement of OAEs

The methods for OAE generation and its measurement have been described elsewhere.⁶⁾ Briefly, animals were anesthetized with urethane (1.5 g/kg, i.p.) and the cochlea was exposed ventrolaterally. A glass-coated platinum microelectrode was inserted into a small hole at the scala media of the 2nd or 3rd turn of the cochlea, and an AC was injected through this electrode to an indifferent electrode in the subcutaneous tissue at the neck. A pure tone was produced in the ear canal by a condenser earphone hermetically supported with the ear bar of a stereotaxic apparatus. Sounds in the ear canal were picked up with a half inch condenser microphone (Brüel & Kjaer 4134) with a probe tube which was inserted into the ear canal and Fast-Fourier-transformed with a digital spectrum analyzer (Advantest, TR 9403) to obtain a frequency-spectrogram. The spectrograms were consecutively obtained 64 times and averaged to improve the S/N ratio. Amplitude of the sound spectra was expressed in dB SPL (re 20 μ Pa).

This OAE measurement was performed using 64 cochleae of the 51 control animals, 53 of the 36 Nitromin-applied animals, and 9 of the 8 kanamycin-treated animals.

2.3 Histochemical Examination of the Cochlear Hair Cells

For examination of the OHC damage, the hair cells were dyed histochemically¹⁴⁻¹⁶⁾ and a surface specimen of the organ of Corti was prepared.¹⁵⁻¹⁸⁾

After measurement of the AC-induced OAEs, each animal was deeply anesthetized and the bilateral temporal bones were quickly removed. A medium composed of 0.05% nitro blue tetrazolium (NBT, Sigma Chemical Company), 0.2 M di-sodium succinate hexahydrate (sodium succinate, Kanto Chemical Co., Inc.), and 0.2 M phosphate buffer (pH 7.4) was immediately perfused into each cochlea from the round window to a hole opened at the apex, and the cochleae were incubated at 37.5°C for 15 min. NBT is a colorless artificial electron acceptor but turns into a blue and insoluble formazan on reduction. Sodium succinate is a substrate of succinate dehydrogenase (SD) which is one of the dehydrogenases in the electron transport system of the mitochondria. The activity of dehydrogenases is directly related to the energy production of the cell. By intracochlear perfusion of this medium, NBT and sodium succinate penetrate into the mitochondria of the hair cells. Then, if SD is active, it will dehydrogenate sodium succinate, and the liberated hydrogen ion will reduce NBT to the blue formazan. Thus, this method makes the cell shape and enzymatic activity in the hair cells visible. Therefore, the morphological change and functional disorder of the hair cells can be examined together with the formazan so produced. After the reaction, for fixation of the cochlear tissues, the cochleae were perfused by 10% formaldehyde in 0.2 M phosphate buffer (pH 7.4) and kept in it at room temperature for one day. The bony shell and the spiral ligament of the cochlea were carefully removed, portions of the organ of Corti were then taken systematically in order from the apex to the basal turn, and surface specimens of the organ of Corti were prepared throughout all turns. The hair cell damage was examined under a microscope and represented schematically as a cochleogram. In the present study, the hair cell damage was conveniently classified into three stages, *i.e.*, 1) slight damage: swelling of the hair cells, 2) moderate damage: change in the cell shape or partial loss of the hair cells, and 3) severe damage: loss of the hair cells. In the cochleogram, grades of the slight, moderate, and severe damages were represented by shaded, meshed, and black bars, respectively.

3. RESULTS

3.1 OAEs in the Control Animals

When an AC was injected through the micro-

Fig. 1 OHCs and AC-induced OAEs in a control animal. **A:** cochleogram for representing the hair cell damage. Grades of the slight, moderate, and severe damages are represented by shaded, meshed, and black bars, respectively. IHC, OHC1, OHC2, and OHC3 indicate the row of the inner hair cells, the 1st, 2nd, and 3rd rows of the outer hair cells. E indicates an AC injection site. **B:** photograph of the hair cells (magnification, 500 $\times$) surrounding E at its centre. **C:** frequency power spectrogram of sounds emitted to the ear canal during combined stimulation of the AC (Fc) of 40 μ A at 0.31 kHz to the 2nd cochlear turn and the tone (Ft) of 80 dB SPL at 1.4 kHz to the ear canal in this figure, Figs. 2, 3, and 4E. The panel configuration is the same in this and the subsequent figures up to Fig. 3. The noise floor of the recording system is indicated by the dotted area in this and the subsequent spectrograms. This noise floor changed little in the post-mortem state.

electrode inserted into the scala media of the 2nd turn and a pure tone was produced simultaneously in the ear canal, faint sounds at frequencies of the AC (fundamental emission, Fc in Fig. 1C) and intermodulation distortion products (distortion emissions, $Ft \pm Fc$ in Fig. 1C) were emitted in the ear canal. In the present study, the fundamental emission was obtained from all the cochleae (64) tested in the control animals and the distortion emissions were observed in 58 cochleae (90.6%). In 6 cochleae from which the distortion emissions could not be detected (9.4%), the holes for the electrode insertion were so large that endolymphorrhea occurred.

The second harmonic emission was observed frequently and the higher one rarely. Similarly, the intermodulation distortion emission of $Ft \pm Fc$ was observed frequently, as mentioned above. In contrast to the psychological cognition of the $2f_1 - f_2$ combination tone due to two primary tones (f_1, f_2), we could not observe the $2Fc - Ft$ emission, probably, because of lack of the corresponding high order distortion term in the nonlinearity to produce the distortion. The AC-induced emissions of the fundamental, the harmonic and the intermodulation distortion products developed linearly with increase in AC strength at least up to 60 μ A, as has been described by Murata *et al.*⁶⁾ Injection beyond 80 μ A often damaged the cochlear function irreversibly. Although we did not try the overdose injection beyond this technical limitation systematically, we believe the intensity function may depart from the linear growth and bend to saturate, when the AC strength exceed a certain threshold.

Cessation of the blood circulation caused immediate decrease in amplitudes of the emissions, then the amplitudes recovered for about 10 min. After this rapid phasic change, the emissions again decreased slowly and the distortion emissions could be observed for about 90 min after the death and the fundamental one for about 2 h, as was described by Murata *et al.*,⁶⁾ although the duration of the post-mortem emissions depended on their frequencies.

In the control animals, all the hair cells produced blue formazan (Fig. 1B) and did not show any pathological changes except for slight damage of a few OHCs near the apex by trepanation and intra-cochlear perfusion of the reaction medium (Fig. 1A).

Fig. 2 Slight damage to OHCs and AC-induced OAEs. Nitromin (20 mg/kg) was applied intraperitoneally and the experiment was performed 7 days after the application. **A:** OHCs of the 1st row were slightly damaged from the base beyond the AC injection site. **B:** OHCs showed a cell-swelling and decrease of production of formazan. **C:** the fundamental emission (Fc) and the distortion emissions (Ft±Fc) were smaller than those in Fig. 1C.

Fig. 3 Severe damage to OHCs and AC-induced OAEs. Nitromin (40 mg/kg) was applied intraperitoneally and the experiment was performed 3 days after the application. **A:** OHCs were severely damaged in all three rows beyond the AC injection site. **B:** OHCs were lost, whereas all IHCs were intact. **C:** the fundamental emission (Fc) was smaller than that in Fig. 1C and no distortion emissions were detected.

3.2 OAEs in the OHC-damaged Animals

In Nitromin- or kanamycin-treated animals, although a susceptibility to these agents differed from animal to animal, the OHC damage progressed in order from the basal to apex turn and from the 1st to 3rd row. In Fig. 2, Nitromin application damaged slightly only the 1st row of OHCs beyond the AC injection site at the 2nd turn. The amplitude of the fundamental emission (Fc) and the distortion

emissions (Ft±Fc) in Fig. 2C were smaller than that in Fig. 1C in spite of the same stimulation. Even if OHCs kept their shapes, the decrease in amplitude of the emissions was often observed when enzymatic activities in OHCs were reduced. In contrast, when the OHC damage was severe, and OHCs were lost completely from the basal turn beyond the AC injection site along all three rows (Fig. 3A), a residual emission still remained at the

Fig. 4 OAEs induced by AC injection from two different sites using the damaged cochlea. Kanamycin (400 mg/kg/day) was applied intramuscularly for 18 days. **A**: cochleogram. **B** and **C**: photographs of the hair cells surrounding the AC injection sites at the 3rd (**B**) and 2nd (**C**) turns, respectively (magnification, 500 $\times$). **D** and **E**: frequency power spectrograms of the emitted sounds due to AC injection to the cochlea and a pure tone stimulation. The AC (**Fc**) of 40 μ A at 0.17 kHz was injected at the 3rd turn with the tone (**Ft**) of 80 dB SPL at 1.0 kHz in **D**, and the AC of 40 μ A at 0.31 kHz at the 2nd turn with the tone of 80 dB SPL at 1.4 kHz in **E**.

frequency of the AC but the distortion emissions disappeared (Fig. 3C).

In Fig. 4, an animal was treated with kanamycin for 18 days and an AC was injected at the 2nd or 3rd turn with a pure tone stimulus. When the AC was injected at the 3rd turn, where OHCs were damaged moderately along the 1st row and slightly along the 2nd and 3rd rows (Fig. 4A and B), the fundamental emission and the distortion emissions could be observed, but their amplitude was smaller (Fig. 4D)

than that of the control animals. These emissions disappeared (Fig. 4E) when, in the same cochlea, the AC was injected at the 2nd turn, where all OHCs were completely lost (Fig. 4A and C).

These results showed that the amplitude decrease of the AC-induced OAEs depended on the extent of the OHC damage, especially at the AC injection site.

3.3 Effect of OHC Damage on Frequency-response of the Fundamental Emission

When the frequency of the AC was changed, keeping its current strength constant at 40 μ A, the frequency-response curves of the fundamental emission from different animals were superimposed in Fig. 5. In the control animals (solid curves), the amplitude of the fundamental emission changed with the frequency of the AC. The curves were parallel to each other and showed a common frequency specificity for the 2nd turn injection (Fig. 5A) and the 3rd turn injection (Fig. 5B), respectively. The best frequency was 2–3 kHz for the 2nd turn injection and 0.7–1 kHz for the 3rd turn injection.

In animals whose OHCs were lost at the AC injection site, characteristics of the frequency-response curves of the fundamental emission (broken curves in Fig. 5) were different from those of the control animals. The amplitude of the fundamental emission was smaller than that of the control animals and became almost constant irrespective of the frequencies of the AC.

The frequency-response curves of the fundamental emission for the 3rd turn injection were compared among animals with variously damaged OHCs in Fig. 6. The shape of the frequency-response curves changed depending on the extent of the OHC damage. As the OHC damage became severe, the amplitude of the emission and its frequency dependence became small and the frequency-response curves became flat.

3.4 Effect of OHC Damage on Frequency-response of the Distortion Emissions

In the control animals, the frequency (**Ft**) of the stimulating tone was changed, leaving its tone level, the frequency and the current strength of simultaneously injected AC were unchanged, and the amplitude of the distortion emissions was plotted against **Ft** in Fig. 7. The amplitude changed with **Ft**, and the emitted frequency range depended on the AC injection site like the frequency-response of the

Fig. 5 Frequency-response curves of the fundamental emission from control animals (solid curves) and those of Nitromin-treated animals (broken curves). OHCs of the Nitromin-treated animals were completely lost at the AC injection site. The AC at 40 μ A was injected at the 2nd (A) and 3rd (B) turns. The noise level of the recording system is indicated by the dotted area in this and the subsequent graphs.

Fig. 6 Effect of different damage to OHCs on frequency-response curves of the fundamental emission. Left panels: cochleograms. OHCs were damaged differently by applications of kanamycin (400 mg/kg/day) for 14 (A), 16 (B), and 17 days (C), respectively. Right panel: frequency-response curves of the fundamental emission in response to an injection of the AC at 40 μ A at the 3rd turn in animals A (solid curve), B (broken curve), and C (dotted curve), respectively.

Fig. 7 Frequency-response curves of the distortion emissions from control animals. The frequency of the tone (F_t) was changed, leaving the tone level (80 dB SPL), the frequency and current strength (40 μ A) of a simultaneously injected AC unchanged. The frequency (F_c) of the AC was 0.31 kHz for the 2nd turn injection (A) and 0.17 kHz for the 3rd turn injection (B), respectively.

Fig. 8 Effect of different damage to OHCs on frequency-response curves of the distortion emissions. Left panels: cochleograms. OHCs were damaged differently by applications of kanamycin (400 mg/kg/day) for 14 (A) and 16 (B) days, respectively. Right panel: frequency-response curves of the distortion emissions in animals A (solid curves) and B (broken curves). The frequency of the tone (F_t) was changed, leaving the tone level (80 dB SPL) unchanged. The frequency (F_c) and current strength of the simultaneously injected AC into the 3rd turn were 0.17 kHz and 40 μ A, respectively.

fundamental emission (Fig. 5).

The frequency specificity of the distortion emissions was also changed by the extent of the OHC damage (Fig. 8). When the OHC damage at the AC

injection site became severe, the amplitude of the emissions and its frequency dependence became small. Moreover, as OHCs at the AC injection site were damaged severely, the emitted frequency range

of the distortion emissions became narrow, and finally, unlike the fundamental emission, the distortion emissions completely disappeared.

4. DISCUSSION

4.1 Selective Damage to OHCs

Since a single shot application of Nitromin damages OHCs, leaving the inner hair cells (IHCs) and other tissues intact,^{19,20)} we utilized Nitromin chiefly for the OHC damage. We could produce also various damaged degrees of OHCs by changing the amounts of Nitromin. Because selective damage to OHCs by kanamycin has been well established,^{18,21-25)} the OHC damage induced by Nitromin was compared with that induced by kanamycin (Figs. 2-4). There were essentially no differences between both the agents in the OHC damage and its effects on the OAEs.

Hair cell damage was examined by preparation of a surface specimen of the organ of Corti with the histochemical dyeing,¹⁴⁻¹⁸⁾ because both the morphological and functional changes of the hair cells can be easily examined at the same time by dyeing due to the production of blue formazan as Akiyoshi *et al.* described.^{15,16)} In the control animals, all the hair cells were dyed evenly throughout the cochlea except for a few OHCs at the uppermost apical end and hair cell damage was not observed at the AC injection site (Fig. 1B). These observations confirmed that an AC injection of at least up to 40 μ A did not change the hair cells at all, either morphologically or functionally. As OHCs were thus tolerant to the AC injection, we could ignore a damage by the AC and consider the OHC damage and its effect on OAEs to be due solely to the ototoxic agents.

4.2 Effect of OHC Damage on OAEs

In the present study, the AC-induced OAEs could be observed at a very high incidence in the control animals (Fig. 1C). Our previous studies showed that these OAEs are not an artifact generated from the measuring system but a biological phenomenon of a cochlear origin.^{5,6)} However, the source of the emissions in the cochlea was not yet clear. It is well established that isolated OHCs showed a motile response to electrical stimulation but other cells in the organ of Corti, including IHCs, did not respond to it.⁷⁻¹⁸⁾ The facts suggest that an electric current injected into the cochlea can induce a motile response of OHCs

in vivo and that the motility causes a mechanical vibration of the basilar membrane, a little part of which is transmitted backward and emitted to the ear canal: OHCs may act as an electromechanical transducer in the cochlea.^{11,26-28)} In the present study, only OHCs were damaged to varying degrees without change in other tissues. Therefore, as shown in Figs. 2-4, the changes in the emissions due to ototoxic agents might relate to the OHC damage. This standpoint *in vivo* coincides well with the facts that, *in vitro*, the electrical motile response could be observed solely in OHCs and not in other cells.

Amplitude of the emissions was decreased by the OHC damage and the decrease depended mainly on the damage of OHCs at the AC injection site (Figs. 2-4). Without recognizable change in cell shape, the decrease of the emissions in amplitude was accompanied by reduced enzymatic activities in OHCs, and this reveals that the normal metabolic condition of OHCs is essential for the generation of the AC-induced OAEs. It is supposed that the motility of OHCs is inhibited by the metabolic depression of OHCs due to ototoxic agents, and that the motility inhibition results in decrease in the basilar membrane vibration, hence decrease in the emission.

When an AC was injected with a simultaneous acoustic stimulation, the fundamental emission and intermodulation distortion emissions were recorded. The mechanical vibration of the basilar membrane, which might be caused electromechanically by the AC as proposed above, may interfere with the traveling wave due to the airborne sound to generate the intermodulation distortion products.^{4,6)} When the OHC damage at the AC injection site became severe, amplitude of the distortion emissions decreased (Figs. 2-4), similarly to the fundamental emission due to the electromechanical transduction. Therefore, OHCs may closely relate to the generation of the distortion emissions. Since the fundamental emission and distortion emissions seemed to change in parallel with each other corresponding to the OHC damage, the distortion decrease may be due to decrease in the fundamental itself. However, the distortion emissions were more vulnerable to various inhibitors of the normal cochlear metabolism and influenced more sensitively by the olivocochlear bundle than the fundamental emission.^{5,6)} In addition, the distortion producing process was more susceptible to the OHC damage than the electromechanical transducing process (Fig. 3). A nonlinearity

to produce this distortion may be due to nonlinear vibration of the basilar membrane described by Rhode.^{29,30)}

When an AC was injected into a region where OHCs were completely lost, the fundamental emission still remained slightly (Fig. 3). In preparations with severe OHC damage from the basal to apical turn, the residual emission could be observed, so far as some OHCs at the uppermost cochlear turn remained normal. The remaining OHCs may contribute to generation of the residual emission. If so, current flows through the remaining OHCs far from the injection site should be evaluated as to whether the current is strong enough to evoke the motile response of these OHCs. Mountain and Hubbard²⁷⁾ showed that the current strength through OHCs just at the AC injection site was reasonably large. At present, the space constant for the current spread from the injection site to the uppermost turn along the cochlea is not yet available. Alternative explanation is possible for the faint residual emission after almost total loss of OHCs. There may be another structure in the cochlea driven electromechanically and it is tolerant to the ototoxic agents. This second electromechanical transducer might be more insensitive to electricity than the OHC-transducer, and the activity of the second one would be masked by the OHC-transducer as long as OHC is normal.

4.3 Frequency-response of OAEs

As was observed in the control animals (Figs. 5 and 7), the AC-induced OAEs had frequency specificity and showed the injection site-dependent best frequency. We proposed a model in the preceding paper⁶⁾ in order to explain the frequency specificity of the AC-induced OAEs. According to the model, the AC-induced vibrations of the fundamental and generated distortion products in the cochlea are constant, respectively, regardless of the AC-frequency. However, because of a filter characteristic for the vibration transmission from the generation site to the ear canal, the amplitude of OAEs is changed frequency-dependently. The frequency characteristics of the emitted higher ($F_t + F_c$) and lower ($F_t - F_c$) sidebands due to a couple of F_t and F_c differed from each other in Figs. 7 and 8. The sideband amplitudes were plotted against F_t frequency and at each frequency of F_t , the frequency of the higher sideband was always larger than that of the lower one. From the viewpoint of the model,

the amplitudes of the both emissions would not usually equal, but differ from each other.

The current flow through the hair cells may be the largest at the AC injection site and the largest motile response of OHCs may be induced at the same site. In addition, the sound-induced motility of isolated OHCs was reported to show frequency-specificity.³¹⁾ Therefore, the maximum emission may be obtained when the frequency of the AC coincides with the best frequencies of the travelling wave tuning and of the sound-induced motility of OHCs at the AC injection site. As the extent of the OHC damage became severe, the maximum of the frequency-response curves became small and their frequency dependence was obscured depending on the OHC damage at the AC injection site (Figs. 6 and 8). Emission decrease in amplitude of the response curve tended to occur from the high frequency end (Fig. 6). This seems to be due to the mode of the OHC damage by the ototoxic agents: the damage progressed from the basal to apical turn. This parallelism between the OHC damage and the curve change also supports our proposal that OHC is responsible for the AC-induced OAEs together with the coincidence of the injected AC frequency with the two best frequencies mentioned above.

In conclusion, the present study linked OHCs with the generation of the AC-induced OAEs *in vivo*: the AC-induced OAEs were decreased by selective damage to OHCs and the decrease depended on the morphological change and, in particular, on the enzymatic activity of OHCs at the AC injection site regardless of existence of IHCs. It is suggested that OHC acts as an active vibrator in the cochlea and is responsible for generation of the AC-induced OAEs, and that production of the intermodulation distortion is due to the nonlinear vibration of the basilar membrane.

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