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# Cochlear outer hair cell electromotility can provide force for both low and high intensity distortion product otoacoustic emissions

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**Abstract**

It is generally believed that the force for the otoacoustic emission (OAE) generation is provided by a mechanism of electromotility, observed in isolated cochlear outer hair cells (OHCs). OHC electromotility is resistant to several ototoxic reagents, it does not depend on ATP hydrolysis, but it can be blocked by specific sulfhydryl reagents: *p*-chloromercuriphenylsulfonic acid (pCMPS) and *p*-hydroxymercuriphenylsulfonic acid (pHMPS). We have used these reagents to test whether they also affect OAE. Application of pCMPS and pHMPS on the round window membrane of anesthetized guinea pigs produced a dose-dependent inhibition of the cubic ($2F_1-F_2$) distortion product OAE (DPOAE). The inhibition developed progressively from high to low frequencies, reflecting the diffusion of the drugs through the cochlear compartment. The effect of pCMPS and pHMPS was different from the effects of furosemide and lethal anoxia, which impair cochlear function but do not block OHC electromotility. pHMPS suppressed DPOAE completely at all sound intensities tested (45–80 dB SPL), whereas furosemide or lethal anoxia caused DPOAE to disappear at low-level stimulation (45–60 dB SPL) only. Our results suggest that the OHC electromotility might provide the force for DPOAE generation not only at low, but also at high stimulus intensities. 

*Key words:* Cochlear amplifier; Outer hair cell motility; Otoacoustic emission; Sulfhydryl reagent; Guinea pig

## 1. Introduction

It was proposed fifty years ago that the extraordinary sensitivity and the frequency selectivity of the mammalian hearing depend on a cycle-by-cycle amplification of sound-induced vibrations in the organ of Corti (Gold, 1948). It is a common assumption that the sound emitted by the cochlea, named otoacoustic emission (OAE) (Kemp, 1978; Zurek, 1981), is a by-product of this amplification (Patuzzi and Robertson, 1988; Dallos, 1992). There are good reasons to suggest that the force for cochlear amplification and for associated OAE is produced by cochlear outer hair cells (OHCs) (Brownell and Kachar, 1985; Brownell, 1990; Schrott et al., 1991). Among cells of the organ of Corti, only the OHCs have been shown to produce active mechanical displacements and therefore may generate sound. OHCs posses an ATP independent (Kachar et al., 1986) mechanism for electromechanical transduction (electromotility) that can operate at acoustic frequencies (Kachar et al., 1986; Ashmore, 1987; Gale and Ashmore, 1997). Also, a significant number of hearing impairments of peripheral origin involves damage of the OHCs (see: Harrison, 1988; Schrott et al., 1991) and absence of OAE (see: Probst, 1990).

There is a discrepancy between the vulnerability of OAE in vivo and the robust nature of the OHC electromotility in vitro. Chronic treatment with aminoglycoside antibiotics suppresses the OAE (Brown et al., 1989). Hypoxia for a few minutes inhibits severely OAE (Kemp and Brown, 1984; Whitehead et al., 1992b; Rebillard et al., 1993). Suppression of the endocochlear potential by the loop diuretics furosemide or ethacrynic acid also inhibits OAE (Kemp and Brown,

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1984; Ueda et al., 1992; Whitehead et al., 1992b; Mills et al., 1993; Mills and Rubel, 1994). In contrast, the OHC electromotility was observed in vitro even several hours after animal death (Brownell et al., 1985; Kachar et al., 1986). Ototoxic reagents like aminoglycosides and loop diuretics do not affect OHC electromotility (Dulon et al., 1989; Kalinec and Kachar, 1993). Salicylates are the only reagents that have been reported to suppress both OAE in vivo (McFadden and Plattsmier, 1984; Kemp and Brown, 1984; Fitzgerald et al., 1993; Kujawa et al., 1992; Ueda et al., 1996) and OHC electromotility in vitro (Shehata et al., 1991; Dieler et al., 1991; Tunstall et al., 1995). The molecular basis of OHC electromotility is thought to be an unidentified voltage-sensitive 'motor' protein embedded in the OHC lateral plasma membrane (Kalinec et al., 1992). Presumably, these proteins are targeted by the mercurial sulfhydryl reagents which effectively block OHC electromotility (Kalinec and Kachar, 1993). Whether the sulfhydryl reagents affect OAE is not known.

On the other hand, if one considers the OAE evoked only by high intensity stimuli, then its physiological vulnerability becomes comparable to the robust nature of the OHC electromotility in vitro. For example, distortion-product OAE (DPOAE) evoked by low stimuli intensity is suppressed by furosemide, ethacrynic acid and shortly after animal death, whereas the DPOAE evoked by high stimuli intensity remains almost unaffected in any of these cases (Mills et al., 1993; Mills and Rubel, 1994; Whitehead et al., 1992b). Somewhat different physiological vulnerability of OAE at low and high intensities has been reported also for transiently evoked OAE (Ryan and Kemp, 1996; Ueda et al., 1996).

The observation that DPOAE evoked by high intensity stimuli does not disappear after animal death led others to speculate that this DPOAE arises from passive mechanical processes that do not utilize metabolic energy (Brown, 1987; Norton and Rubel, 1990; Whitehead et al., 1990). This is why studies relating OHC damage and DPOAE were usually restricted to studies of DPOAE evoked by low intensity stimulation (Schrott et al., 1991). Several authors named the DPOAE evoked by high intensity stimulation as 'passive' DPOAE in contrast to the DPOAE evoked by low intensity stimulation which they defined as an 'active' DPOAE (for example see: Mills et al., 1993; Mills and Rubel, 1994). However, there are indications that 'passive' DPOAE does depend on some type of metabolic energy. It slowly disappears within several hours after animal death and can be affected post-mortem by acoustic trauma (Whitehead et al., 1992b). It seems possible that the robust phenomenon of OHC electromotility can provide the force for DPOAE generation at high stimulus intensities even after animal death. We tested this by using two mercurial sulfhydryl reagents that inhibit OHC electromotility: *p*-chloromercuriphenylsulfonic acid (pCMPS) and *p*-hydroxymercuriphenylsulfonic acid (pHMPS). We analyzed the effects of pCMPS and pHMPS on the cubic ($2F_1-F_2$) DPOAE in guinea pig in comparison to the effects of furosemide and lethal anoxia.

## 2. Materials and methods

### 2.1. Animals and surgical procedures

Adult pigmented guinea pigs of either sex weighing between 250 and 500 g were anesthetized with xylazine 5.0 mg/kg and ketamine 35 mg/kg, i.m. The anesthesia was maintained by additional half-dose injections every hour. Body temperature was maintained at 38°C by an electric thermal pad. All the experiments were done in a sound proof acoustic chamber (IAC, NY). The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985).

A head holder secured the guinea pig's head in place. The pinna was removed to facilitate fitting of the OAE probe. Using an operating microscope, the external ear canal was examined to assure that it was free from debris and that the tympanic membrane was intact and clear. A disposable otoscopic speculum was trimmed and placed into the outer ear canal using a flexible arm. An E-type neonatal dual channel probe (Otodynamic Limited, UK) was introduced into the plastic speculum and acoustically sealed with an Ear-Link foam block (Cabot Safety Corporation, IN). The cochlea was surgically exposed through a dorso-lateral opening of the bulla, preserving the tympanic membrane and ossicles intact. Then the wound was closed with a wet cotton strip to preserve humidity in the middle ear cavity and to reduce the acoustical noise. After control DPOAE measurements, the middle ear cavity was re-exposed, a small drop of sulfhydryl reagent solution was applied to the round window and the bulla was resealed with a cotton strip. These manipulations were performed without changing the position of the head or of the probe in the outer ear canal. When the testing of the reagent effect in one ear was finished, DPOAE amplitudes were measured in the contralateral ear to insure that the effect of sulfhydryl reagent was local.

### 2.2. Application of pCMPS and pHMPS

50–100 mM pHMPS or pCMPS (Sigma, MO) were applied in 2–3-μl drops to the round window membrane using a Microloader (Eppendorf, Germany) held by a micromanipulator. Assuming the total volume of cochlear fluids in the guinea pigs to be about 18 μl (16 μl

perilymph, 2 μl endolymph) (Salt and Thalmann, 1988), the applications of 2–3 μl at 50–100 mM solutions should produce a maximal concentration of 5–17 mM inside the cochlea after diffusion. We expect, however, this concentration to be significantly less, because of perilymph flow, leakage of test solution from the round window, and diffusion of the drugs into adjacent tissues. Therefore, we studied mainly the effects of the drugs on the DPOAE generated by cochlear regions close to the round window (i.e. high-frequency regions), where the drug concentration should be the highest. The effects of pCMPS were tested in 13 guinea pigs and of pHMPS in 23 guinea pigs.

In control experiments (5 animals), physiological saline was applied to the round window. Then, changes of DPOAE amplitude did not exceed 5 dB within two hours after saline application.

### 2.3. Furosemide and lethal anoxia

We studied the effects of furosemide (6 guinea pigs) and lethal anoxia (3 guinea pigs) on DPOAE amplitudes. The furosemide solution (50 mg/ml, Sigma, MO) was injected intraperitoneally. Lethal anoxia was produced by inhaling of $CO_2$ for 5–6 min.

### 2.4. Otoacoustic emission

Using the Otodynamic Analyzer (Otodynamics, UK), we recorded otoacoustic emission at the frequency $2F_1-F_2$, produced by two simultaneously presented tones with frequencies $F_1$ and $F_2$ (cubic DPOAE). Two types of measurements were made: (i) the amplitude of DPOAE, evoked by two equilevel stimuli ($L_1 = L_2 = 50$, 60, or 70 dB SPL) with the frequency ratio ($F_2/F_1$) maintained at 1.22, was measured as a function of the frequency $F_2$ within the range of 1–6 kHz; (ii) the relationship between the amplitude of DPOAE ($2F_1-F_2$, $F_2/F_1 = 1.098$) and the stimulus intensity, within the range of 45–80 dB SPL, was tested at the frequency $F_2 = 6$ kHz. These parameters were determined through preliminary experiments. They represent a compromise between the requirement of relatively large DPOAE amplitudes (the amplitude of 'active' DPOAE is maximal at $F_2/F_1 = 1.22$, $L_1/L_2 = 10$–20 dB (Whitehead et al., 1995a,b)), and the requirement that the transition between 'active' and 'passive' DPOAEs has to be at the intensities around 65 dB SPL, well below the upper limit of our sound-generating system (80 dB SPL). The latter requirement dictates $F_2/F_1$ and $L_1/L_2$ to be far from the optimal values (Whitehead et al., 1992a).

DPOAE responses were evaluated at 5-min intervals for up to 2 h post application. For control values, 3–5 measurements usually were done before administration of the drug or initiating the anoxia. To plot the time course of the drug effect, we expressed the changes of DPOAE amplitudes in decibels relative to the control values.

To compare the changes in the putative active and passive DPOAEs, we plotted the calculated normalized values against the respective noise floor levels (Fig. 4). The 'noise floor' was determined as the distortion products measured two hours after the animal's death. Thus, the 'noise floor' values in our experiments represented a sum of distortions due to several factors: acoustic noise, distortions of sound generating and measuring system, and probably some residual DPOAE which might be present two hours after animal death at high stimulus intensities. These 'noise floor' values were well below DPOAE measurements at all stimulus intensities (Fig. 3), allowing us to trace the effect of different reagents on DPOAE amplitude relative to noise floor. DPOAE values after drugs or anoxia measured relative to the noise floor were expressed as a percentage of the corresponding control values. The statistical significance of DPOAE changes has been estimated with Student's *t*-test.

## 3. Results

### 3.1. Effects of sulfhydryl reagents on DPOAE

Application of pCMPS on the round window membrane produced a gradual decrease of DPOAE, starting

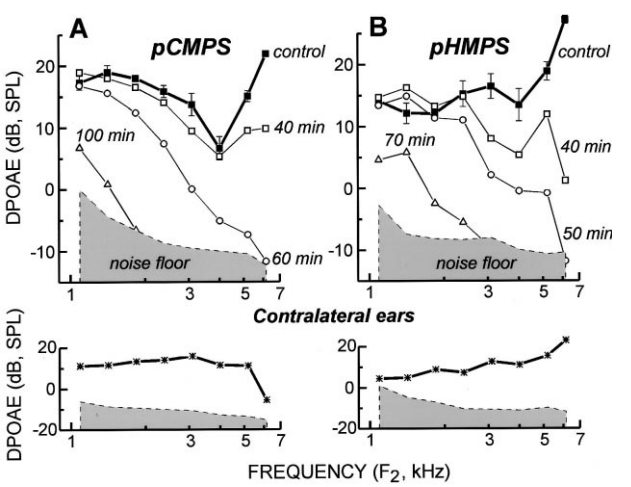


Fig. 1. Suppression of cubic DPOAE ($2F_1-F_2$) by pCMPS (A) and pHMPS (B). The relationships between the stimulus frequency ($F_2$) and the DPOAE amplitude are shown before the round window membrane application of the drugs (thick lines, the average of four measurements) and after indicated time intervals (thin lines with different symbols). Three μl of 100-mM test solutions was applied in A and B. Error bars represent standard errors. Bottom panels show DPOAE in the contralateral (untreated) ears after the testing of the effects of the drugs has been finished. Shaded areas on all the panels show the averaged noise estimations. DPOAE has been evoked by two tones with equal intensity ($L_1 = L_2 = 60$ dB SPL) and with frequency separation of $F_2/F_1 = 1.22$.

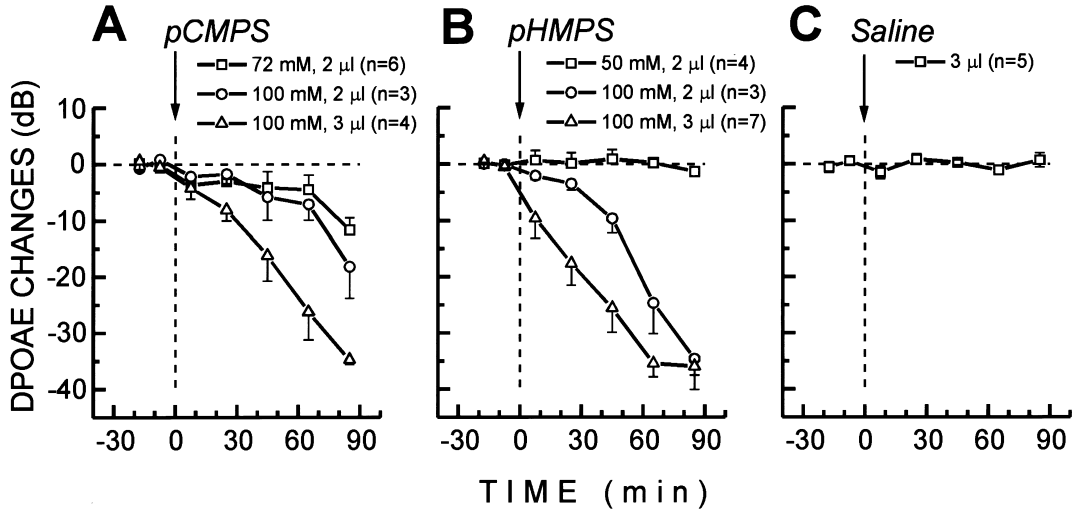


Fig. 2. Time courses of DPOAE changes after round window membrane application of pCMPS (A), pHMPS (B), and physiological saline (C). Changes of DPOAE at $F_2 = 6$ kHz were expressed in decibels relative to the mean control values. The zero in each time scale corresponds to the moment of drugs or of saline application. Legends show doses and number of animals in each group. Error bars represent standard errors. Stimuli intensity: $L_1 = L_2 = 60$ dB SPL; frequency separation: $F_2/F_1 = 1.22$.

at high frequencies and propagating slowly to lower frequencies. In the typical experiment shown in Fig. 1A, the high-frequency DPOAE started to decrease 15 min following application and dropped below noise floor within 100 min. Low-frequency DPOAE was affected only after high-frequency DPOAEs had been significantly reduced. pHMPS produced qualitatively the same effect (Fig. 1B). All effects were local, since the DPOAE in the contralateral, untreated ears was not affected (Fig. 1, bottom panels).

The effects of both sulfhydryl reagents were dose-dependent (Fig. 2A,B). pHMPS suppressed the emission faster than pCMPS did (Fig. 2A,B). This difference is consistent with the greater ability of pHMPS to penetrate through biological membranes (Rothstein, 1970). Both reagents applied at relatively high doses (3 µl of a 100-mM solution) completely inhibited DPOAE at $F_2 = 6$ kHz within one hour, while the same amount (3 µl) of physiological saline gave practically no effect (Fig. 2C).

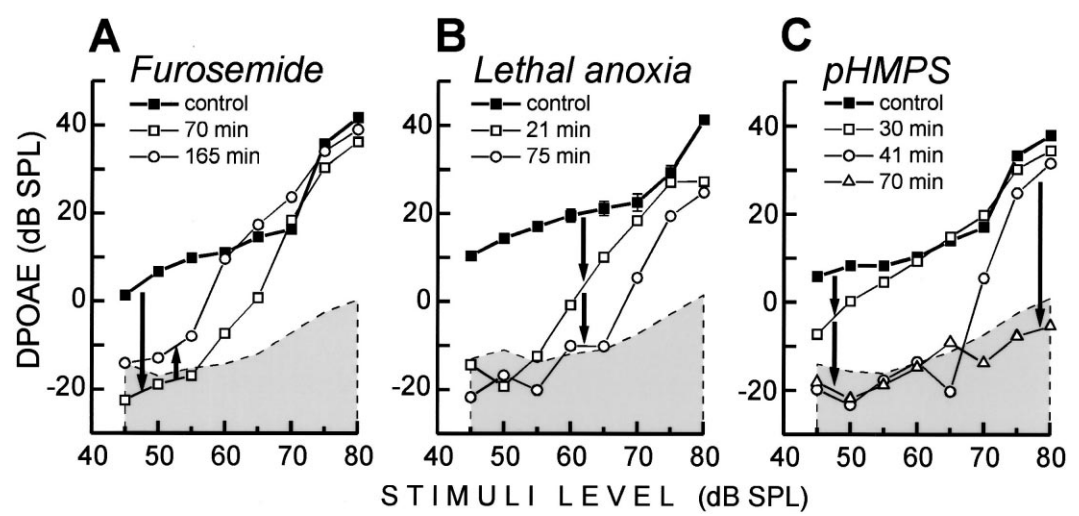


Fig. 3. Comparison of the effects of furosemide (A), lethal anoxia (B), and pHMPS (C) on the DPOAE input/output relationships at $F_2 = 6$ kHz. Four measurements before drugs or $CO_2$ application formed the control curves (thick lines); the thin lines with different symbols show the input/output relationships after indicated time intervals. Error bars represent standard errors. Furosemide has been injected intraperitoneally (120 mg/kg), pHMPS has been applied on the round window membrane (100 mM, 3 µl). Stimuli frequency separation ($F_2/F_1 = 1.098$) has been optimized to obtain optimal distinction between low- and high-level DPOAEs (see text). Shaded areas show the noise estimations.

### 3.2. Comparison of the effects of furosemide, lethal anoxia, and pHMPS on DPOAE input/output functions

The DPOAE input/output relationships clearly showed two phases of growth (Fig. 3): an initial slow growth at low stimulus levels (45–70 dB SPL), and a steeper growth at higher stimulus levels (70–80 dB SPL). These two phases were prominent at the stimulus frequency ratio of $F_2/F_1 = 1.098$. In correspondence with previously reported data on gerbils (Mills et al., 1993; Mills and Rubel, 1994), furosemide abolished the DPOAE evoked by low-intensity stimuli in our experiments, whereas it did not affect the DPOAE evoked by high-intensity stimulation (Fig. 3A). The furosemide effects on the DPOAE evoked by low-intensity sound stimulation (Fig. 3A) required a dose of 120 mg/kg. We did not observe any statistically significant effects on DPOAE using furosemide at 60 mg/kg. The furosemide suppression of the active DPOAE was maximal at one hour of the i.p. injection, followed by a gradual recovery thereafter. It was more prominent at high frequencies than at low frequencies. Following the definition of Mills and Rubel (1994), we could designate the DPOAE evoked by low-intensity stimuli as an 'active' emission and the DPOAE evoked by high-intensity stimulation as a 'passive' emission.

Additional evidence for the different vulnerability of DPOAE evoked by low- and high-intensity stimulation was obtained in the experiments with lethal anoxia. Immediately after the death of the animal the low-level ('active') DPOAE fell below the noise floor, but the high-level ('passive') DPOAE was recorded after death (Fig. 3B), decreasing slowly, to be abolished not until two hours after death (Fig. 3B). These findings are consistent with the data of Whitehead et al. (1992b).

In contrast to the effects of furosemide and short-term lethal anoxia, the application of pHMPS to the round window membrane suppressed not only DPOAE evoked by low-level stimulation, but also the DPOAE responses to high-intensity stimulation. The pHMPS effect developed rapidly at high frequencies. In the typical experiment illustrated on Fig. 3C, the DPOAE elicited by 45–65 dB SPL stimulation disappeared within 40 min after pHMPS application, while the DPOAE evoked by stimuli at higher intensity was suppressed later. Such complete disappearance of high-level ('passive') DPOAE was never observed in furosemide experiments and it occurred in the lethal anoxia experiments only hours after the death of the animal.

To compare the effects of drugs and of lethal anoxia on DPOAEs evoked by low- and high-intensity stimuli, we normalized the values of DPOAE amplitudes to the corresponding noise values estimated after the death of the animal (Fig. 4). It was found that the effects of furosemide or short-term lethal anoxia on the DPOAEs evoked by low- and high-intensity stimulation are different ($P < 0.05$). Meanwhile no statistical significance was found between the DPOAEs to low- and high-intensity stimuli after pHMPS or 2 h after death (Fig. 4).

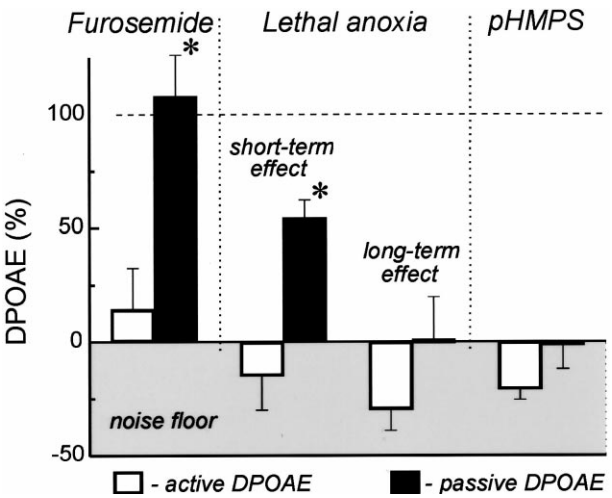


Fig. 4. Comparison of the action of furosemide, lethal anoxia, and pHMPS on the 'active' DPOAE evoked by low-intensity stimulation (45–60 dB SPL, white bars) and on the 'passive' DPOAE evoked by high-intensity stimulation (70–80 dB SPL, black bars). Bars show the DPOAE amplitudes relative to the noise floor of experimental setup, after drugs or lethal anoxia, calculated as a percentage of the DPOAE values in the control period. Noise floor of experimental setup was determined from the noise readings given by ILO-system after the animal death. The negative percentage means that DPOAE amplitudes fell below the noise floor of our experimental setup and we are not able to estimate the statistical significance of any difference of the DPOAE amplitudes in this area. Mean values and standard errors are shown. Doses and number of animals in the groups: furosemide (120 mg/kg, $n = 3$), lethal anoxia ($n = 3$), pHMPS (100 mM, 3 μl, $n = 5$). The DPOAE measurements taken 30–60 min after drug application were averaged in the case of furosemide and pHMPS. Bar 'short-term effect' of lethal anoxia represents the average data 10–20 min after death of animal, 'long-term effect' of lethal anoxia 2 h and more after death. Asterisks designate the statistical significance of the differences between 'active' and 'passive' DPOAEs ($P < 0.05$).

## 4. Discussion

This study shows that sulfhydryl reagents (pCMPS and pHMPS) suppress DPOAE and that their effects are different from the effects of furosemide or short-term lethal anoxia. This is significant, because the sulfhydryl reagents affect OHC electromotility (Kalinec and Kachar, 1993), while furosemide and short-term anoxia do not (Kalinec and Kachar, 1993; Kachar et al., 1986). A complete suppression of the DPOAE evoked by high-intensity stimulation does not occur after furosemide administration or immediately after the death of the animal, but was observed after pHMPS treatment (Figs. 3 and 4). Previously, only combined treatments with aminoglycosides and ethacrinic acid was reported

to suppress 'passive' DPOAE (Whitehead et al., 1992b). This treatment is known to completely destroy OHCs (Russell et al., 1979; Hayashida et al., 1989; Hiel et al., 1992). Our data also show that the DPOAE evoked by high-intensity stimulation, or 'passive' DPOAE can be suppressed in a living animal. If the OHC electromotility is required for the generation of high-intensity DPOAE, then the suppression of high-intensity DPOAE with pHMPS (Figs. 3 and 4) is probably due to an action of this reagent on the OHC electromotility. The term 'passive' DPOAE can therefore be misleading.

Indeed, our results support this point of view. First, DPOAE disappeared only on the side of sulfhydryl reagent application, and therefore the effect cannot be attributed to the penetration of the reagents to the blood or perilymph. Second, the sulfhydryl reagents produced a gradual decrease of DPOAE starting from the high frequencies (Fig. 1), which is consistent with a slow diffusion of the reagent inside the cochlea. Finally, pHMPS was able to suppress DPOAE to high-intensity stimulation (Figs. 3 and 4), while the drop of endocochlear potential after furosemide (Fig. 3A), or lethal anoxia (Fig. 3B) failed to suppress it.

Prior to this study, only salicylates have been reported to inhibit both the OHC electromotility in vitro (Shehata et al., 1991; Dieler et al., 1991; Tunstall et al., 1995), and the OAEs in vivo (McFadden and Plattsmier, 1984; Kemp and Brown, 1984; Fitzgerald et al., 1993; Kujawa et al., 1992; Ueda et al., 1996). The effect of salicylates in vivo was reported to be similar to the effect of diuretics: they suppress DPOAE evoked by low-intensity stimuli, leaving DPOAE evoked by high-intensity stimulation unchanged (Kujawa et al., 1992). In vitro, the suppression of OHC electromotility by salicylates is not complete, and partly attributed to the loss of the OHC turgor (Kakehata and Santos-Sacchi, 1996). In contrast, the inhibition of OHC electromotility by pHMPS or by pCMPS is not accompanied by the loss of cell turgor (Kalinec and Kachar, 1993). The difference in effects of salicylates and sulfhydryl reagents on the high-intensity DPOAE is probably related to more specific blocking of the OHC motile mechanism by sulfhydryl reagents.

It has been reported that ATP, ATP-gamma-S, and cibicron, an ATP antagonist, suppress cubic DPOAE evoked by low-intensity stimulation (Kujawa et al., 1994a; Kujawa et al., 1994b). It is possible that this suppression results from the effect of these drugs on the OHCs (Housley et al., 1992; Skellett et al., 1997). However, ATP does not inhibit OHC electromotility but, on the contrary, enhances it (Housley et al., 1995) as it also does not inhibit the high-intensity DPOAE (Kujawa et al., 1994a).

It has been a commonly held point of view that two-tone acoustic distortions at the high-stimulus intensities arise from the passive mechanical non-linearities of the cochlear tissues (Brown, 1987; Norton and Rubel, 1990; Whitehead et al., 1990; Kujawa et al., 1992). From this point of view one could argue that pHMPS suppresses high-intensity DPOAE because it might change the mechanical properties of cochlear tissues. Consistently, one would also claim that these changes account for the effects evoked by combined aminocyclo-side/ethacrinic acid treatment and by post-mortem effects two hours after death. However, the effect of acoustic trauma on the high-intensity post-mortem DPOAE (Whitehead et al., 1992b) remains unexplained. The possibility that OHC electromotility provides force for both low- and high-intensity DPOAE generation provides a more tenable explanation of the observed phenomena. The high-intensity DPOAE decreases gradually post-mortem reflecting the deterioration of OHC force-generating mechanism. Indeed, it was reported that some active mechanical tuning in the organ of Corti is still present immediately after animal death and gradually deteriorates within several hours post-mortem (ITER, 1989; Flock et al., 1997).

pCMPS and pHMPS are non-diuretic reagents specific to the SH-group (Rothstein, 1970). They may affect SH-groups in a functionally important region of different proteins and, therefore, may impair cochlear function in different ways. For example, it is known that apart from the direct effect on the OHC electromotility (Kalinec and Kachar, 1993) these sulfhydryl reagents are able to inhibit the endocochlear potential (Thalmann et al., 1977). It is assumed that at least three processes are essential for the functioning of the cochlear amplifier and for the generation of the associated OAEs: (i) the maintaining of the positive endocochlear potential; (ii) the mechanoelectrical transduction in the OHCs; (iii) the active force generation by the OHCs. The inhibition of any one of these processes would result in the suppression of OAEs. Therefore, the inhibition of low-intensity DPOAE by pHMPS is not surprising. On the other hand, the suppression of high-intensity DPOAE by pHMPS together with the resistance of this emission to furosemide or lethal anoxia (Fig. 3) indicate that high intensity sounds may directly evoke OHCs motility even in the absence of an endocochlear potential. Therefore, OHC motility may provide force for the DPOAE generation even at high stimulus levels.

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