NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
Annual Technical Report
1996

Submitted by

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Project Director

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United States Army Medical Research and Development Command
(Walter Reed Army Institute of Research)

and

Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 6 of 9

Protecting the Auditory System and Prevention of Hearing Problems

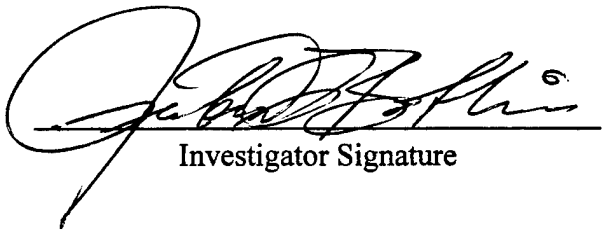
Project Directors:
Richard Bobbin, PH.D.
Charles Berlin, Ph.D.

ANIMAL USE
SEPTEMBER 20, 1995 THROUGH JULY, 1996

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Protecting the Auditory System and Prevention of Hearing Problems**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC#
Guinea Pig	276	83	1061



Investigator Signature

Volume 6 Protecting the Auditory System and Prevention of Hearing Problems

Project Directors: Richard Bobbin, Ph.D.
Charles Berlin, Ph.D.

Participating Scientists: Sharon Kujawa, Ph.D.
Carlos Erostegui, M.D.
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Chen C, Nenov A, Bobbin RP (1995) Noise exposure alters the response of outer hair cells to ATP. *Hear Res* 88:215-221.

Kujawa SG, Fallon M, Bobbin RP (1995) Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. *Hear Res* 85:142-154.

Kujawa SG, Fallon M, Skellett RA, Bobbin RP (1996) Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. II. Influence of local calcium-dependent mechanisms. *Hear Res*. In Press.

Bobbin RP (1996) Chemical receptors on outer hair cells and their molecular mechanisms. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 29-55.

Skellett RA, Crist JR, Fallon M, Bobbin RP (1996) Chronic low-level noise exposure alters distortion product otoacoustic emissions. *Hear Res*. In Press.

Skellett RA, Fallon M, Cullen JK, Bobbin RP (1996) "Conditioning" the auditory system with continous vs. interupted noise of equal acoustic energy: Is either exposure more protective? *Hear Res*. In Preparation.

Chen C, LeBlanc C, Bobbin RP (1996) Failure to detect an ATP-evoked current response in outer hair cells of rat cochlea. *Hear Res*. Submitted.

Berlin CI, Hood LJ, Hurley AE, Wen H, Kemp DT (1995) Binaural Noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise. *Hear Res* 87:96-103.

Berlin CI, Hood LJ, Hurley AE, Wen H (1996) Hearing aids: only for hearing impaired patients with abnormal otoacoustic emissions. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 99-111.

Hood LJ, Berlin CI, Hurley AE, Wen H (1996) Suppression of otoacoustic emissions in normal hearing individuals. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 57-72.

Skellett RA, Crist JR, Fallon M, Bobbin RP (1996) Chronic low-level noise exposure alters distortion product otoacoustic emmissions. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, Abstract 126, February 4-8.

Skellett RA, Fallon M, Bobbin RP (1996) "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, Abstract 136, February 4-8.

Chen C, LeBlanc CS, Bobbin RP (1996) ATP does not generate a current response in outer hair cells of rat cochlea. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, Abstract 526, February 4-8.

I. ABSTRACT: (SPECIFIC AIMS) (Maximum of 200 words)

ANIMAL PROJECT: The **SPECIFIC AIMS** of this study are to demonstrate and explore mechanisms for preventing the effects of intense sound. In years 01, and 02 we discovered that continuous, ipsilateral primary stimulation (CM-LIPS) will produce complex changes in the mechanics of the cochlea, possibly related to "toughening". In year 03 we discovered that ATP is involved in generating this complex mechanics. We extended the noise exposure studies and found that continuous noise is less effective than interrupted noise in inducing "toughening". Cellular mechanism studies discovered that ATP kills outer hair cells in vitro and in vivo, indicating that ATP may be a key player in noise-induced deafness and "toughening".

HUMAN PROJECT: We have found that binaural noise suppresses linear click evoked emissions twice as much as ipsilateral noise and 3 times as much as contralateral noise. However, subjects with noise exposure often show poor or reduced emissions when the stimulus is a click. Of 20 subjects enrolled and 13 completely tested so far in the multi-day protocol, subjects with noise exposure effects at higher frequencies show MORE suppression at and around 1500 Hz than do subjects with no hearing loss. This may support the original hypothesis in this program that "Noise Tender Ears" will show different emission suppression patterns from ears that are tough.

II. INTRODUCTION (Hypothesis): The following text is approximately the same as in the Progress Reports for year # 01 and # 02. The nature of the problem that this proposal addresses is that soldiers may be exposed to intense noise hazards which will affect their hearing. The hypothesis to be tested is that noise induced hearing loss can be prevented or attenuated.

The **animal experimental literature** demonstrates that chronic low level sound will "toughen" ears so subsequent intense noise will induce less damage (e.g., Canlon et al., 1988; Campo et al., 1991; Franklin et al., 1991). One hypothesis regarding this phenomena is that low level sound activates inhibitory cholinergic efferents that reduce the effects of intense sound by attenuating cochlear partition motion. Alternatively, there may be down-regulation of a protein that has a role in noise induced hearing loss. ATP may be released together with ACh from the efferents. In years #02 and #03 we have obtained data indicating that the purinergic neurotransmitter, ATP, may play a major role in noise effects in the cochlea because we demonstrated that the ATP receptor on OHCs may be down-regulated during noise exposure (Chen et al., 1995). ATP may be a major player in the effects of toughening because it has powerful effects on the OHCs and supporting cells and ATP is known to induce cell death (Bobbin, 1996; Eybalin, 1993; Kujawa et al., 1994b; Valera et al., 1994). If it can be shown that ATP induces cell death in the cochlea then evidence for a role for ATP in noise-induced hearing loss will be obtained.

The **human experimentation program** was proposed to develop emission-based tests to detect abnormal cochlear function rapidly and accurately (Berlin, 1995; Berlin et al., 1993). The procedure offers the equivalent of a non-invasive acoustic microscope to analyze the integrity of the OHCs; this is an important tool since OHC damage is always seen in humans who have suffered noise damage. The hypothesis is that "noise tender" ears that are particularly susceptible to noise

damage will show different emission suppression patterns from ears that are "tough".

Our intention remains to study 100 musicians and industrial workers who have had matched exposures to noise in an efferent suppression paradigm. Before starting the final design of this study we laid basic parametric groundwork relative to the nature of the stimuli, their delivery (whether clicks or tones, whether binaural, contralateral only, or ipsilateral), the echo analysis, and whether there are any pertinent gender and ear differences which have to be entered into the final subject selection.

In summary, in **animals** we will test the hypothesis that: (1) the impact of noise on hearing can be lessened; (2) certain classes of drugs may prevent (or exacerbate) noise-induced hearing loss. In **human subjects**, we will explore the concept that some individuals are more or less susceptible to noise damaging effects. We will describe those populations to determine the basis of this "toughness" or susceptibility to damage from noise. We will examine whether the techniques which we may discover aid in preventing noise-induced hearing loss in soldiers.

III. BODY (STUDIES AND RESULTS CONDUCTED UP TO AND DURING THE CURRENT BUDGET YEAR 03):

ANIMAL PROJECT: In years #01, #02, and #03 we have continued to carry out acute and chronic experiments. In brief, the **acute** experiments focus on the effects of ipsilateral sound on the mechanics of the cochlea, and studies of single cells in the whole cell voltage clamp configuration; and the **chronic** experiments examine "toughening" in the intact animal. These experiments follow directly our stated specific aims for years #01, #02 and #03 and the first three TECHNICAL

OBJECTIVES: to extend studies which demonstrate that contralateral, ipsilateral, or "toughening" sound will prevent the effects of intense noise, to test the role of the efferents, and to explore cellular mechanisms.

The methods were described (Kujawa et al., 1992; 1993; 1994a; year #01 progress report; Appendix 2 and 3). Briefly, guinea pigs are anesthetized (urethane: 1.5 gm/kg) and tracheotomized. ECG is monitored and temperature maintained at 38° ± 1°C. The right auditory bulla is exposed, opened and tendons of the middle ear muscles are sectioned. For drug application to the cochlea, holes are placed in the cochlear basal turn: one in scala tympani for the introduction of perfusates and one in scala vestibuli to allow fluid escape. Perfusates are introduced into scala tympani at approximately 2.5 µl/min for 10 min through a pipette coupled to a syringe pump. DPOAEs ($2f_1$-f_2 & f_2-f_1) are recorded in response to primary stimuli (f_2/f_1=1.2) delivered to the right ear of each animal by an acoustic probe/hollow ear bar assembly. Acoustic signals present within the canal are detected by a microphone system (Etymotic Research, ER-10) contained within the probe. Microphone output is directed via a preamplifier (Etymotic Research, ER-1072) to a signal analyzer (Hewlett Packard 3561A). To extract the DPOAE from the canal spectrum, the signal is sampled, digitized and submitted to Fast Fourier Transform (FFT) analysis. The resulting spectrum is averaged (over 25 samples) and displayed at the spectrum analyzer (1 kHz window, 3.75 Hz BW). DPOAE amplitude is defined as the spectral peak corresponding to the DPOAE frequency. The contralateral stimulus is a wideband noise with an overall level of 70 dB SPL and flat from 0.9 to 15.8 kHz.

The **acute** experiment examined the influence of continuous, moderate-level (60 dB SPL) ipsilateral primary stimulation (CM-LIPS) on the f_2-f_1 DPOAE at 1.25 kHz (quadratic). The

stimulation and response monitoring protocols consisted of quiet for 15 min, then turning the primaries on for 9 min, off for 1 min and then on for an additional 3 min. CM-LIPS resulted in a complex change in the magnitude of f_2-f_1 (see Appendix 2 and 3 for publications). In previous years, we ruled out the efferent nerves as contributors to CM-LIPS (year #01, Kujawa et al., 1995; Appendix 2) and described the role of calcium (year #02, Kujawa et al., 1996; Appendix 3). In year #03 we continued to explore mechanisms for the CM-LIPS-induced alteration in cochlear mechanics. We have made a major discovery: PPADS, an ATP antagonist, potentiates the decrease in the quadratic DPOAE during CM-LIPS. ATP suppresses the decrease. Suramin, probably acting as an ATPase inhibitor, acts similar to ATP. Our new hypothesis based on these observations is that ATP is slowly released during CM-LIPS and produces the changes observed in the quadratic DPOAE. This is in harmony with results in year #02 indicating that there is an apparent down-regulation of ATP receptors during "toughening" (Chen et al., 1995; Appendix 1).

In year #03, in the **chronic** experiment, we used a "toughening" noise that was more intense (than the 65 dB SPL rms, A-scale used in year #02; Skellett et al., 1996a; Appendix 5) in order to test whether toughening occurs with the intense sound. In addition, we compared the effects of continuous vs. interrupted exposures of equal acoustic energy (experiment #3 in the original proposal). The results show that although both exposures studied had equal acoustic energy, the interrupted noise exposure was significantly less damaging to the cochlea than the continuous noise. The results are being prepared for publication (Skellett et al., 1996b; Appendix 6). In addition, we tested whether these sounds induce toughening as monitored by a decrease in the effects of a subsequent intense sound exposure. The results suggest that overall, there were significant differences in the degree of protection provided by prior exposure to moderate-level continuous vs.

interrupted toughening sounds. There was protection afforded by the interrupted noise protocol, however, the frequency region where protection occurred was limited to the region above that of the noise exposure band. Such results have not been reported previously and may lead to new understanding of the toughening phenomena. There was a lack of protective effect across the entire test frequency range when the continuous noise protocol was used as the moderate-level toughening exposure. The results are being prepared for publication (Skellett et al., 1996b; Appendix 6). In the following year we will extend these findings by examining for differences in the ATP receptors of animals treated by this toughening treatment.

To examine the cellular basis for these "toughening"-induced changes in the cochlea (experiment #4 in original application), we carried out whole cell voltage clamp recordings from the OHCs (Chen et al., 1995). During year 03 we completed the demonstration that rat OHCs do not respond to ATP but that both rat and guinea pig supporting cells do respond to ATP. Interestingly, the rat is less sensitive to noise-induced hearing loss, possibly indicating that the rat is already toughened. A manuscript describing these results has been submitted for publication (Chen et al., 1996; Appendix 7). These results are a totally new discovery. During year #04, we will compare noise induced hearing loss and toughening in the rat to the guinea pig.

During year #03, we examined the effect of ATP on short term cultured guinea pig and rat OHCs. Here, we have made an additional major discovery: acute application of ATP to these OHCs from guinea pig, but NOT RAT, induced a loss of fluid and turned the cells into membrane ghosts. We extended this finding in vivo. Survival of the animal for two weeks after one half hour of ATP administration to the cochlea was found to result in the appearance of OHC ghosts upon subsequent histology. Thus we have formulate the following hypothesis: that intense sound releases excessive

ATP and this kills the OHCs of the cochlea; toughening exposures result in a down regulation of ATP receptors and subsequently less cell death due to intense sound exposure.

HUMAN PROJECT: All of the items listed in last year's progress report have been initiated or completed. These include building and completing the single pulse capability of our Echo Lab system, developing norms for tone bursts and narrow bands of noise, and beginning age and gender norm collection. The single pulse paradigm, unavailable on any commercial instrument, was essential here in order to avoid contamination with ipsilateral efferent suppression in which the first click in a train interferes with the amplitude of the emissions obtained from the subsequent clicks.

We have made the following important progress:

1. Two versions of our unique and proprietary Lab View system have been built and are operational collecting data in binaural, ipsilateral and contralateral modes in a single-pulse transient-evoked format not otherwise possible.

2. Internal reliability of data collection over a three month period in excess of 0.92.

3. Right vs. Left ear and gender differences are present but only occasionally reach statistical significance (Hood et al. 1996b).

4. Binaural Noise suppresses linear click evoked emissions twice as much as ipsilateral noise and 3 times as much as contralateral noise (Berlin et al., 1995; Appendix 9; confirmed by Liberman et al. 1996). However, subjects with noise exposure often show poor or reduced emissions when the stimulus is a click. Our proprietary system is able to generate transient tone-burst stimuli in singlet form (in contrast to the 4-at-a-time convention which has inherent efferent suppression) and we chose to us a 1500 Hz tone burst after elaborate and extensive pilot testing with various combinations of tones and noises. The basic finding that Binaural noise

suppresses more than ipsilateral or contralateral noise in a forward masking paradigm is found again using the 1500 Hz tone bursts as follows:

Table 1 Time Data 8-18 msecs

Mean Suppression

	Right Ear			Left Ear		
	Binaural	Ipsilateral	Contralat.	Binaural	Ipsilateral	Contralat.
Normal Hearing	-1.321	-0.434	+0.102	-1.170	-0.439	-0.412
High Frequency Loss	-1.561	-0.664	-0.61	-1.179	-0.875	-0.854

In real-world simultaneous noise conditions the effect is at least three to four times more powerful, but the reader should keep in mind that these data are collected in a forward-masking paradigm in which the masker follows the offset of the transient by 10 msecs. There is a trend for the right ear to show more suppression than the left ear which has appeared periodically in all of our experiments to date. We have shown that this effect is related to the menstrual cycle in females, in which the size of the emission fluctuates but not the amount of suppression.

5. This pattern of binaural > ipsi > contra suppression is not seen in the few parents we have studied who carry recessive genes for deafness.

6. Suppression and emission strength vary with age, which has to be factored in when

one uses emission suppression as a tool for auditory system predictions.

7. The best levels of suppression occur at low levels of noise and when signals are below 65 dB SPL ...the cochlear active process (See summary in: Hood et al. 1996a; Appendix 11).

8. Of 20 subjects enrolled and 13 completely tested so far in the multi-day protocol, subjects with noise exposure effects at higher frequencies show MORE suppression at and around 1500 Hz than do subjects with no hearing loss. This may support the original hypothesis in this program that "Noise Tender Ears" will show different emission suppression patterns from ears that are tough.

9. New basic science data from MLS experiments:

The Maximum Length Sequence, a mathematical sequence of clicks designed to stimulate the ear at high rates and deconvolve the resultant responses, predictably shows a diminution of ABR (a neural response) with increased rate from 125.1 to 1000 (Figure 1; Appendix 8), but no loss of cochlear microphonic amplitude even at 1000 clicks per second. However, when the same mathematical system is used to collect otoacoustic emissions in normal subjects, the emission response is drastically reduced in amplitude (Figure 2; Appendix 8). From this we reason that the cochlear microphonic might come from different parts of the hair cell, the former probably from a segment inaccessible to the efferents (the stereocilia or their tip links?) and the latter from the body of the hair cell itself which is accessible to efferent effects. Patients with no operating contralateral efferent system show a rate-related MLS emission suppression only for ipsilateral rate changes, thus supporting our basic hypothesis. This hypothesis and our attendant observations will be expanded upon in the next year.

IV. CONCLUSIONS (PLANS FOR YEAR 04 OF SUPPORT):

ANIMAL PROJECTS: The completed research indicates that ATP is a major neuromodulator in the cochlea, possibly released from the efferent nerve fibers innervating the cells in the cochlea. Furthermore it appears that ATP plays an integral role in cochlea mechanics, in the response of the cochlea to low level sound and toughening, and in noise induced hearing loss. During year #04, we will continue to document the effects of ATP and ATP antagonists on cochlear mechanics and the changes in cochlear mechanics induced by continuous low level sound. We will explore if these ATP induced changes in cochlear mechanics are related to toughening. We will ask if the cochlear mechanics are changed during toughening in a manner that involves ATP receptors. ATP antagonists will be tested against noise induced cochlear damage. In addition, we will continue our studies that indicate ATP administered to short term OHC cultures and into the cochlea induces cell death. ATP antagonists will be used to attempt to block this cell death. In addition, molecular biological tests for apoptosis will be carried out to test whether the ATP induced cell death is apoptotic-like. Finally, experiments will be conducted comparing rat and guinea pig responses to toughening and noise induced cochlear damage to obtain preliminary data regarding the effect of the lack of ATP receptor activation in rat OHCs.

HUMAN PROJECT: We will expand our subject base to include an additional 80 subjects from among servicemen and women and more musicians with matched noise exposure histories. We will compare functions of all available commercial instruments to MLS system now available to help decide which of the instruments has most promise in the assessment of efferent suppression. So far it appears as if the MLS system generates an intrinsic test of efferent function. We will collect

transient evoked emissions in some small animals and then add an animal study to block MLS efferent effects on emission amplitude compared to cochlear microphonic amplitude.

V. REFERENCES:

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Kujawa, S.G., Fallon, M., Skellett, R.A., and Bobbin, R.P. (1996) Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. II. Influence of local calcium-dependent mechanisms. Hear. Res. In Press.

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VI. List of publications and abstracts:

a). Chen, C., Nenov, A., and Bobbin, R.P. Noise exposure alters the response of outer hair cells to ATP. Hear. Res. 88, 215-221, 1995.

b). Kujawa, S.G., Fallon, M., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. Hear. Res. 85: 142-154, 1995.

c). Kujawa, S.G., Fallon, M., Skellett, R.A., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. II. Influence of local calcium-dependent mechanisms. Hear. Res. In Press, 1966.

d). Bobbin, R.P. Chemical receptors on outer hair cells and their molecular mechanisms. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 29-55, 1996.

e). Skellett, R.A., Crist, J.R., Fallon, M. and Bobbin, R.P. Chronic low-level noise exposure alters distortion product otoacoustic emissions. Hear. Res. In Press, 1996.

f). Skellett, R.A., Fallon, M., Cullen, J.K. and Bobbin, R.P. "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Hear. Res. In Preparation, 1996.

g). Chen, C., LeBlanc, C., and Bobbin, R.P. Failure to detect an ATP-evoked current response in outer hair cells of rat cochlea. Hear. Res. submitted, 1996.

h). Berlin, C.I., Hood, L.J., Hurley, A.E., Wen, H., and Kemp, D.T. Binaural Noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise. Hear. Res, 87, 96-103, 1995.

i). Berlin, C.I., Hood, L.J., Hurley, A.E., and Wen, H. Hearing aids: only for hearing impaired patients with abnormal otoacoustic emissions. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 99-111, 1996.

j). Hood, L.J., Berlin, C.I., Hurley, A.E., and Wen, H. Suppression of otoacoustic emissions in normal hearing individuals. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 57-72, 1996.

k). Skellett, R. A., Crist, J.R., Fallon, M., and Bobbin, R.P. Chronic low-level noise exposure alters distortion product otoacoustic emissions. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 126, February 4-8, 1996.

l). Skellett, R. A., Fallon, M., and Bobbin, R.P. "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 136, February 4-8, 1996.

m). Chen, C., LeBlanc, C.S., and Bobbin, R.P. ATP does not generate a current response in outer hair cells of rat cochlea. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 526, February 4-8, 1996.

VII. Inventions and Patents

None

VIII. List of Appendices

Appendix 1. Chen, C., Nenov, A., and Bobbin, R.P. Noise exposure alters the response of outer hair cells to ATP. Hear. Res. 88, 215-221, 1995.

Appendix 2. Kujawa, S.G., Fallon, M., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. I. Response characterization and contribution of the olivocochlear efferents. Hear. Res. 85: 142-154, 1995.

Appendix 3. Kujawa, S.G., Fallon, M., Skellett, R.A., and Bobbin, R.P. Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation. II. Influence of local calcium-dependent mechanisms. Hear. Res. In Press, 1996.

Appendix 4. Bobbin, R.P. Chemical receptors on outer hair cells and their molecular mechanisms. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 29-55, 1996.

Appendix 5. Skellett, R.A., Crist, J.R., Fallon, M. and Bobbin, R.P. Chronic low-level noise exposure alters distortion product otoacoustic emissions. Hear. Res. In Press, 1996.

Appendix 6. Skellett, R.A., Fallon, M., Cullen, J.K. and Bobbin, R.P. "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Hear. Res. In Preparation, 1996.

Appendix 7. Chen, C., LeBlanc, C., and Bobbin, R.P. Failure to detect an ATP-evoked current response in outer hair cells of rat cochlea. Hear. Res. submitted, 1996.

Appendix 8. Figures for the human studies.

Appendix 9. Berlin, C.I., Hood, L.J., Hurley, A.E., Wen, H., and Kemp, D.T. Binaural Noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise. Hear. Res, 87, 96-103, 1995.

Appendix 10. Berlin, C.I., Hood, L.J., Hurley, A.E., and Wen, H. Hearing aids: only for hearing impaired patients with abnormal otoacoustic emissions. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 99-111, 1996.

Appendix 11. Hood, L.J., Berlin, C.I., Hurley, A.E., and Wen, H. Suppression of otoacoustic emissions in normal hearing individuals. In: **Hair Cells and Hearing Aids**. C.I. Berlin, Editor, Singular Publishing Group, Inc. San Diego. p. 57-72, 1996.

Appendix 12. Skellett, R. A., Crist, J.R., Fallon, M., and Bobbin, R.P. Chronic low-level noise exposure alters distortion product otoacoustic emissions. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 126, February 4-8, 1996.

Appendix 13. Skellett, R. A., Fallon, M., and Bobbin, R.P. "Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective? Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 136, February 4-8, 1996.

Appendix 14. Chen, C., LeBlanc, C.S., and Bobbin, R.P. ATP does not generate a current response in outer hair cells of rat cochlea. Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, abstract 526, February 4-8, 1996.

APPENDIX # 1
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HEARING RESEARCH

Noise exposure alters the response of outer hair cells to ATP

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Abstract

The outer hair cells (OHCs) are one target of noise-induced effects. To date there are few studies which examine changes in the function of OHCs induced by noise exposure. There is increasing evidence that ATP may be a neuromodulator acting on OHCs. Therefore, we examined the possibility that the response to ATP may be altered by low-level noise exposure. ATP was tested on cation currents recorded from outer hair cells (OHCs) isolated from chronic noise-exposed guinea pigs and compared to currents recorded from normal control animals. The whole-cell variant of the patch-clamp technique was used. The incidence of response to 100 μM ATP was decreased in OHCs from noise-exposed animals as compared to controls when normal internal and external solutions were employed. When K^+ was substituted by *N*-methyl-glucamine (NMG^+) in the pipette solution, there were significant differences in the magnitudes of ATP-evoked currents between cells from noise-exposed and control animals. This was observed in both normal and 20 mM Ba^{2+} external solutions. In addition, the response to ATP exhibited a dependency on OHC length. In short OHCs (< 65 μm) from noise-exposed animals the magnitude of the response to ATP was significantly reduced. By contrast, the response in long OHCs (> 65 μm) from noise-exposed animals was increased. Results suggest that low-level noise exposure induces changes in OHCs which affect the response of the cell to ATP.

Keywords: Voltage-clamp; Patch-clamp; ATP-gated channel; Cochlea; Noise exposure

1. Introduction

Noise exposure induces several alterations in the structure and function of the cochlea (see review by Saunders et al., 1985). The outer hair cells (OHCs) are one target of noise-induced effects (e.g., Cody and Russell, 1985; Puel et al., 1988; Decory et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Subramaniam et al., 1994). Morphological evidence shows that noise exposure causes: damage to OHC stereocilia, changes in intracellular structures such as mitochondria, and swelling or loss of the OHCs (Saunders et al., 1985). Some of these morphological changes have been correlated with changes in neuronal thresholds (Boettcher et al., 1992) and distortion product otoacoustic emissions (DPOAEs). The latter are thought to reflect the physiological state of the OHCs (Subramaniam et al., 1994).

There are only a few studies which examined the effect of intense sound on the function of the hair cells directly. Decory et al. (1991) showed that isolated OHCs taken from noise-exposed guinea pigs exhibited altered motility and viability. Acoustical-evoked receptor potentials recorded from both inner hair cells (IHCs) and OHCs are altered during and after exposure to intense sound with the OHCs undergoing a sustained depolarization (Cody and Russell, 1985). Some of these effects are probably due to excessive passage of K^+ and Ca^{2+} through the transduction channels of the hair cells together with entrance of Ca^{2+} and Na^+ through voltage-dependent channels. In other systems, it is well known that an increase in the levels of intracellular free Ca^{2+} can initiate events which result in the alteration or death of cells (Berridge, 1994; Morley et al., 1994). Thus there is evidence that the OHCs are excessively depolarized by noise exposure, resulting in altered function and possibly cell death.

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There are a host of molecular events occurring in and around OHCs that could be altered by noise exposure. One possibility is an altered response to neurotransmitter or neuromodulator. Bobbin and Thompson (1978) first proposed ATP as a neuromodulator or transmitter in the mammalian cochlea (see review Eybalin, 1993). Aubert et al. (1994,1995) present data suggesting that ATP may act as a neuromodulator in vestibular organs. ATP and analogues applied into the perilymph compartment exert profound effects on cochlear function, as indicated by an abolishment of compound action potential of the auditory nerve and DPOAEs together with a shift in the summating potential (Bobbin and Thompson, 1978; Kujawa et al., 1994a). Incubation of organ of Corti with ATP agonist analogs increases inositol phosphate accumulation suggesting an activation of this second-messenger system (Niedzielski and Schacht, 1992). Extracellular application of ATP to isolated OHCs of guinea pig depolarizes the cell membrane by inducing non-selective cation currents (Ashmore and Ohmori, 1990; Nakagawa et al., 1990; Housley et al., 1992; Kujawa et al., 1994a; Nilles et al., 1994). Since antagonists also have profound effects on the cochlear potentials, it appears that endogenous ATP may have a role in ongoing physiological mechanisms (Kujawa et al., 1994b). In addition, noise exposure may increase extracellular levels of ATP to such an extent that it contributes to the excessive depolarization and cell death observed.

Thus we initiated studies of the hypothesis that ATP is involved in noise-induced changes to the OHCs. Specifically, in this study we examined whether the response of OHCs to ATP was altered by a low-level, chronic noise exposure. The intensity of the noise was set below the level thought to induce cellular damage (Bohne, 1976). We recorded the cation currents evoked by ATP in isolated OHCs harvested from chronic noise-exposed guinea pigs and compared them to the currents evoked from control animals using the whole-cell configuration of the patch-clamp technique.

2. Methods

2.1. Noise exposure

Guinea pigs ($n = 35$, in groups of 10 or less; age 1–3 months) were placed in a small sound-attenuating booth and exposed to a continuous, moderate-intensity narrow band noise (cutoffs at 1.1 and 2.0 kHz; A-scale, rms: 65 dB SPL) 24 h a day for 10–11 days. Control guinea pigs ($n = 30$) were maintained in an environment of normal noise at the university's animal care facilities. Both groups of the animals were given free access to food and water during the exposure. The care and use of the animals reported on in this study were approved by the Animal Care and Use Committees of the Louisiana State University Medical Center.

Table 1
Composition of solutions (mM)

	Internal		External	
	K^+ sol.	NMG^+ sol.	HBS sol.	Ba^{2+} sol.
NaCl	-	-	137	120
KCl	134	-	5.4	-
$CaCl_2$	0.1	0.5	2.5	-
$BaCl_2$	-	-	-	20
$MgCl_2$	0.5	-	0.5	0.5
CsCl	-	-	-	5
HEPES	5	10	10	10
NMG^+	-	120	-	-
TEA-Cl	-	35	-	-
EGTA	11	11	-	-
Glucose	-	-	10	5
Na_2ATP	2	4	-	-
Na_2GTP	0.1	0.1	-	-
Sucrose	-	10	10	-

HBS: modified Hank's balanced saline; NMG^+: *N*-methyl-glucamine.

2.2. Cell isolation

OHCs from control and noise exposed guinea pigs were acutely isolated as described previously (Erostegui et al., 1994). Briefly, guinea pigs were anesthetized with pentobarbital (30 mg/kg, i.p.) or urethane (1.5 g/kg, i.p.), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS) (Table 1). The bone surrounding the cochlea was removed and the organ of Corti was placed in a 200 μl drop of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then transferred into the dishes containing a 100 μl drop of HBS using a microsyringe, and stored at room temperature until use (within about 4 h). The length of each cell was measured with a calibrated reticulum prior to recording. OHCs were selected for study if they met several morphological criteria (Ricci et al., 1994). No morphological changes of isolated OHCs from noise-exposed animals were observed.

2.3. Whole-cell voltage clamp

Single dispersed OHCs either from noise-exposed guinea pigs or normal control animals were voltage clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with Axopatch-1D and Axopatch-200A patch-clamp amplifiers (Axon Instruments). Patch electrodes were fabricated from borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 kHz (−3dB) using a 4-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using personal microcomputers. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments)

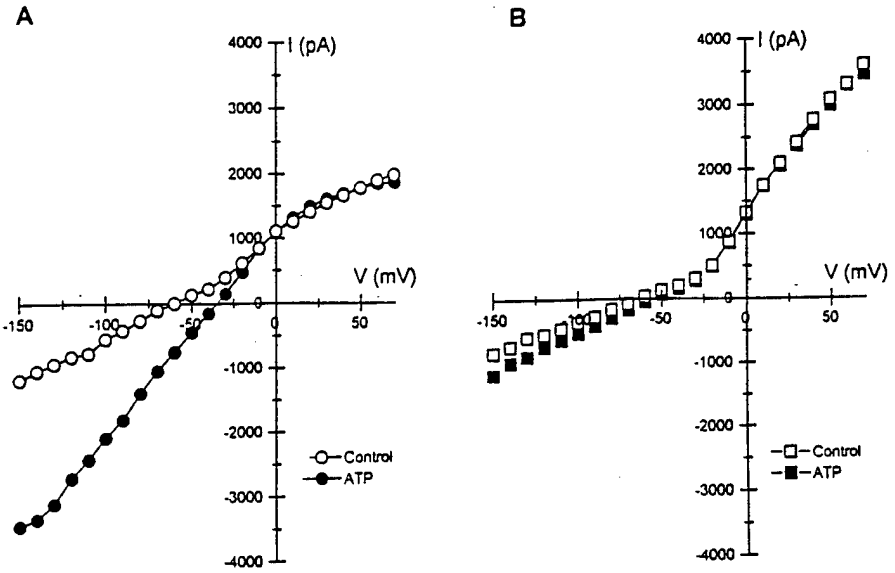

Fig. 1. Typical examples of ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed animal (B). Currents were evoked by 60 ms steps from a holding potential of -60 mV. Normal HBS external solutions together with K^+-containing internal solutions were used. Currents were measured before (○, □) and during (●, ■) application of 100 μM ATP.

using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording, and an 80% series resistance compensation was normally applied. No substraction of leakage current was made.

2.4. Solutions

The composition of the solutions used is shown in Table 1. The HBS solution was utilized for the bath perfusion. ATP (Sigma)-containing external solutions were prepared daily from 100 mM stock solution and was

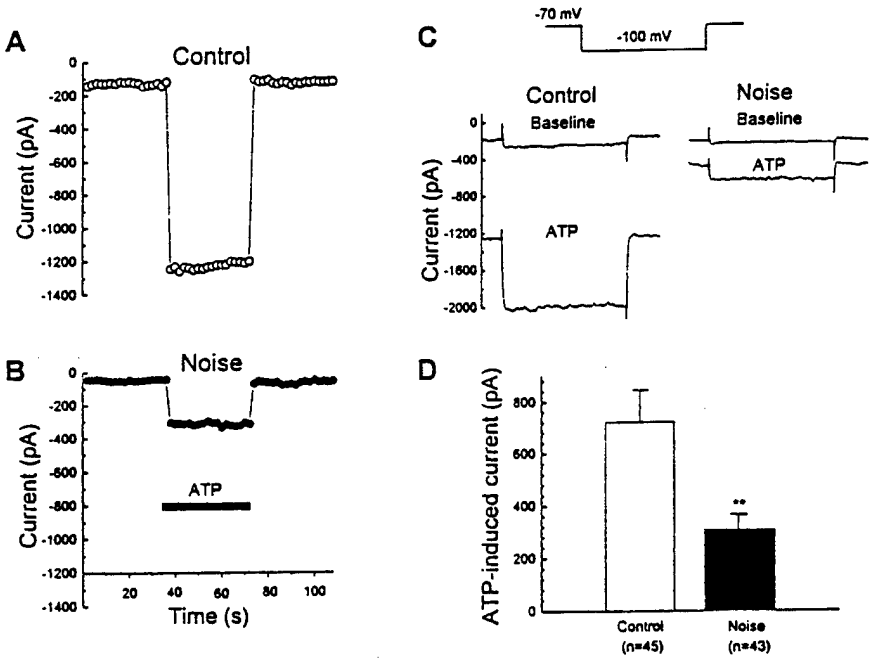

Fig. 2. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and normal HBS external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and a noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP (100 μM)-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in (C). Statistical significance was measured by ANOVA (** $P < 0.01$).

delivered from an U-tubing system as described previously (Erostegui et al., 1994). All the external solutions were adjusted to a pH of 7.40 with NaOH and had a osmolality of 300 mosM. The internal solutions were adjusted to a pH of 7.35 with HCl and had a osmolality of 284 mosM. All experiments were conducted at room temperature (22 ~ 24°C).

Data are presented as means ± SE. Statistical significance was measured by chi-square test and analysis of variance (ANOVA), as appropriate. P values less than 0.05 were considered statistically significant.

3. Results

Fig. 1 illustrates the current–voltage (I-V) relationships in the absence and presence of 100 μM ATP in OHCs from control and noise-exposed guinea pigs using normal K^+ internal and HBS external solutions. Currents were evoked by 60 ms steps from a holding potential of −60 mV. Monitored at −100 mV, the incidence of ATP responses was significantly reduced in cells from noise-exposed animals as compared to the incidence in cells from control animals (Table 2). In addition, the amplitude of the ATP induced inward current at −100 mV in cells from noise-exposed animals showed a tendency to be smaller than those recorded from control animals ($P = 0.06$).

Table 2
Incidence of response to ATP in OHCs from control and noise-exposed guinea pigs

Control		%	Noise-exposed		%
Response *	No Response *		Response *	No Response *	
25	22	53	14	62	18 **

* Number of cells are given.
** Statistical significance was measured by chi-square test ($P < 0.001$).

To further examine this tendency, we isolated the ATP-induced currents by substituting N-methyl-glucamine (NMG^+) for K^+ in the pipette to block K^+ currents while using normal HBS external solution. Under such conditions, the incidence of the response to 100 μM ATP was not significantly different in the two groups of cells (noise: 88%, $n = 49$ vs. control: 98%, $n = 46$). However, the magnitude of 100 μM ATP-induced inward currents in cells from noise-exposed animals was decreased both at the holding potential of −70 mV (Fig. 2A, control; Fig. 2B, noise) and at the step to −100 mV (Fig. 2C control and noise). Examination of the data at −100 mV indicated that the effect was significant (noise: 303 ± 64 pA, $n = 43$ vs. control: 720 ± 125 pA, $n = 45$; $P < 0.01$; the zero change in response to ATP of the non-responders were not included; Fig. 2D).

To avoid the deleterious effects of increased intra-

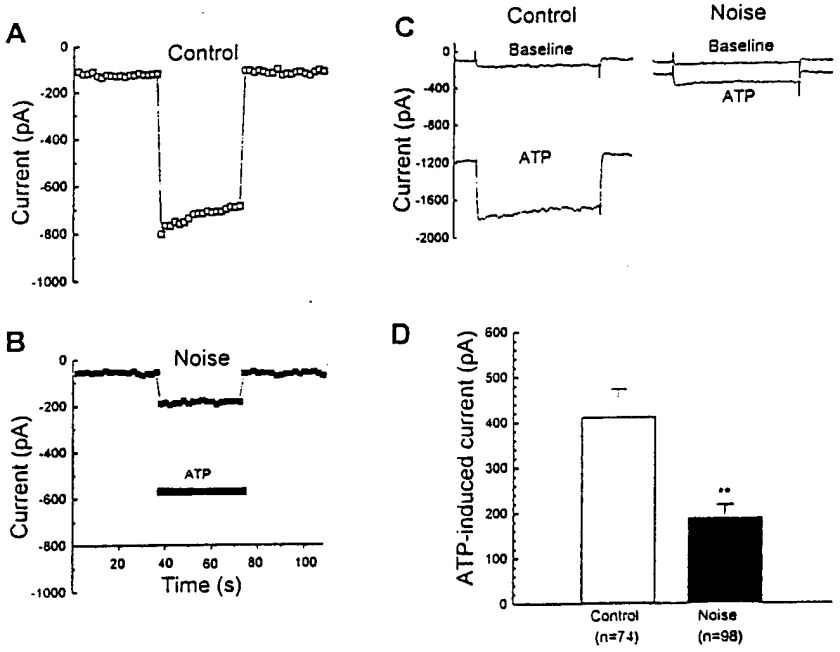

Fig. 3. ATP-evoked current response obtained in external Ba^{2+} solution. A and B: typical examples of ATP (100 μM)-induced inward currents recorded from an OHC taken from a control (A) and noise-exposed animal (B). A holding potential of -70 mV and 20 mM Ba^{2+}-containing external solution together with NMG^+-containing internal solution were used. C: superimposed examples of inward current traces recorded from an OHC taken from a control and noise-exposed animal in the absence and presence of 100 μM ATP. Current was elicited by a 70 ms hyperpolarizing step to -100 mV from a holding potential of -70 mV. D: amplitudes (mean + SE) of ATP-induced inward currents obtained from OHCs taken from control and noise-exposed guinea pigs. Currents were measured at -100 mV using the step protocol in C. Statistical significance was measured by ANOVA (** $P < 0.01$).

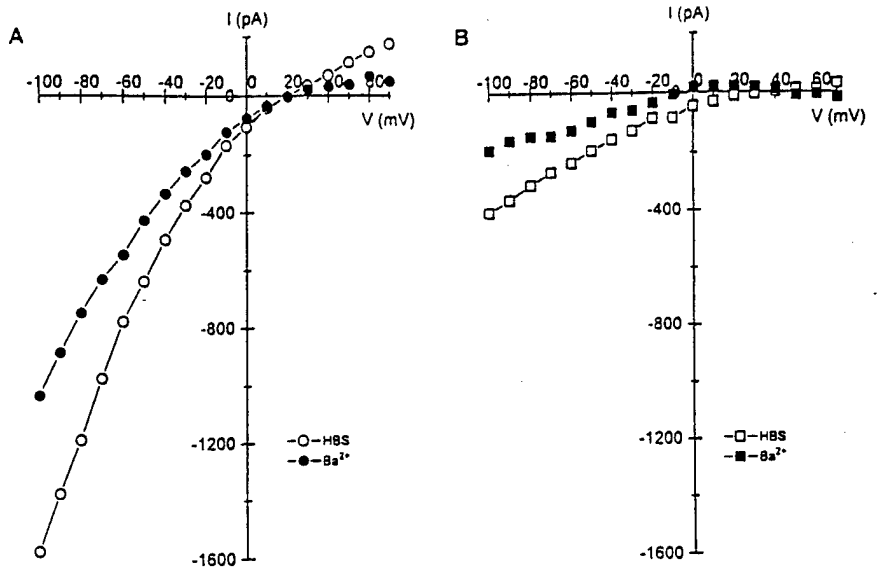

Fig. 4. ATP-evoked current at different voltages. Typical examples of 100 μM ATP-induced effects on *I-V* relationships recorded from an OHC taken from a control (A) and noise-exposed (B) animal. Data shown were obtained utilizing normal HBS external solutions (□), 20 mM Ba^{2+}-containing external solutions (■) and pipettes containing a NMG^{+}-containing internal solution. Plotted are the differences between currents in the presence and absence of the drug at the various test potentials. Currents were evoked by 70 ms steps from a holding potential of -70 mV.

cellular Ca^{2+} caused by ATP and to further block outward K^{+} current, we studied the effect of utilizing Ba^{2+}-containing external solution while keeping the same NMG^{+}-containing solution in the pipette. Some of the cells ($n = 26$, noise; $n = 27$, control) were the same ones studied in the previous paragraph. Results were similar to that using

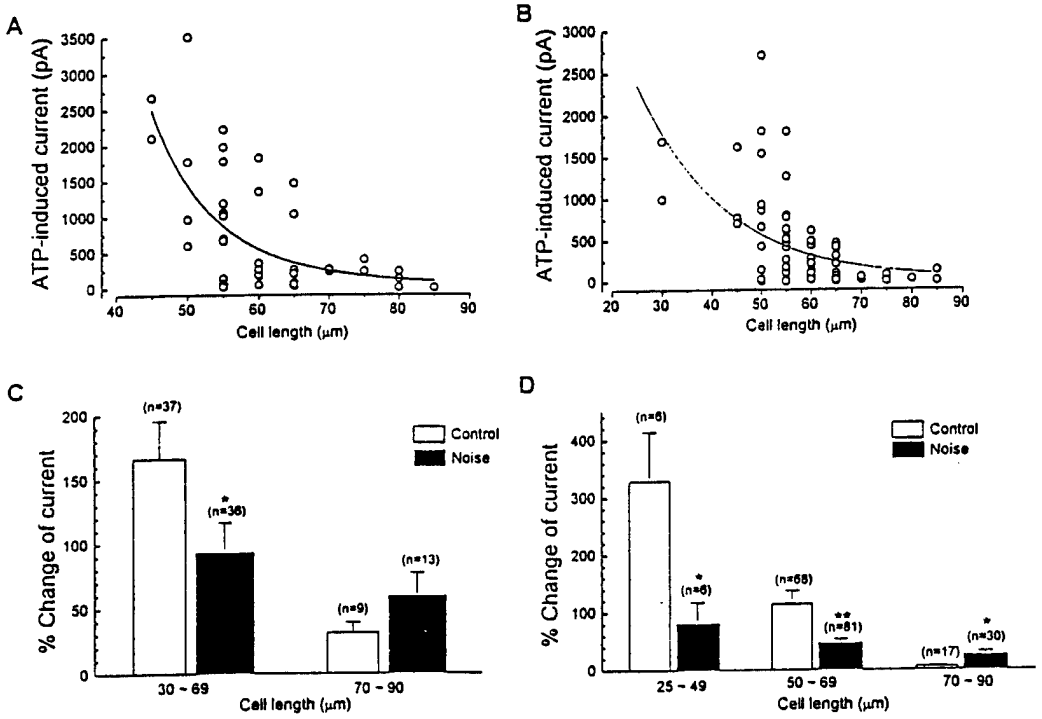

Fig. 5. A: scattergram of the 100 μM ATP-induced currents recorded from OHCs taken from control animals plotted against cell length. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. B: same as in (A) only data obtained utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. C: comparison of the magnitude of responses to 100 μM ATP between OHCs taken from control and noise-exposed guinea pigs. Data obtained utilizing normal HBS external solutions together with NMG^{+}-containing internal solutions. D: same as in (C) only utilizing 20 mM Ba^{2+}-containing external solutions together with NMG^{+}-containing internal solutions. The divisions according to length were arbitrarily made. In some cases the same cell contributed data that appears in both normal and 20 mM Ba^{2+}-containing external solution groups. Each bar is mean + SE. Statistical significance was measured by ANOVA (* $P < 0.05$; ** $P < 0.01$). All current amplitudes were measured at -100 mV using a step protocol consisting of 70 ms hyperpolarizing steps to -100 mV from a holding potential of -70 mV.

HBS external solution. There was no difference between groups in the incidence of the response (noise: 84%, $n = 117$ vs. control: 81%, $n = 91$). However, the magnitude of the ATP-induced currents in cells from noise-exposed animals was decreased both at the holding potential of -70 mV (Fig. 3A, control; Fig. 3B, noise) and at the step to -100 mV (Fig. 3C, control and noise). Examination of the data at -100 mV indicated that the response was significantly different from control (noise: 188 ± 29 pA, $n = 98$ vs. control: 410 ± 62 pA, $n = 74$; $P < 0.01$; Fig. 3D). Fig. 4 shows the *I-V* relationships of ATP-induced inward currents in OHCs from control (Fig. 4A) and noise-exposed (Fig. 4B) animals in HBS and Ba^{2+}-containing external solutions. The amplitudes of the ATP-elicited inward currents were smaller in Ba^{2+}-containing external solution than those in HBS solution. The pattern of results in OHCs from control and noise-exposed animals were similar.

Length of OHCs varies according to their position in the cochlear partition and is related to the function of OHCs (Pujol et al., 1992). We observed a length dependency for the ATP-induced effects in HBS (Fig. 5A) and Ba^{2+}-containing (Fig. 5B) external solutions for OHCs taken from control animals. This result is similar to that reported for acetylcholine-induced effects in OHCs (Erostegui et al., 1994). Fig. 5C (in HBS solution) and Fig. 5D (in Ba^{2+}-containing solution) illustrate the amplitudes of ATP-induced inward currents expressed as a percent of baseline currents (at -100 mV) in cells grouped according to length. The amplitudes of the currents were significantly decreased in the short cells (cell length < 65 μm) from noise-exposed animals as compared to those from controls. In contrast, ATP-induced currents were enhanced in cells with lengths over 65 μm from noise-exposed animals as compared to corresponding cells from controls.

4. Discussion

Results show that there were significant differences in the incidence and magnitudes of cation currents evoked by ATP (100 μM) in OHCs taken from guinea pigs exposed to chronic low-level noise when compared to the currents evoked from cells obtained from control animals. The response to ATP was decreased in short OHCs whereas in longer OHCs the response was increased.

At present we can only speculate as to the structural or biochemical mechanisms underlying our observations. The results are in harmony with the hypothesis that endogenously released ATP may be involved in the effects induced in the cochlea by low-level, chronic noise exposure. In other systems, the chronic release of ATP has been suggested to result in a down-regulation of purinoceptors (Maynard et al., 1992). This mechanism was proposed to explain the altered response to an ATP analogue in the rabbit isolated central ear artery after chronic electrical stimulation of the great auricular nerve (Maynard et al., 1992). By analogy, during noise exposure there may be a continuous exposure of OHCs to a high level of endogenous ATP which induces a similar down-regulation of ATP receptors in short OHCs. An up-regulation of ATP receptors may occur in long OHCs. Only future experiments can determine if such a mechanism accounts for the observed altered responses to ATP.

Alternatively, any noise-induced structural damage may have altered the response to ATP. We did not determine whether damage had occurred in the cochlear tissue we sampled. On the other hand, based on the literature to date, the noise level was probably not sufficiently intense to produce physical damage. No reports were found using the same band and level of noise in guinea pigs. However, the noise is the same level as one of the octave band of noise (0.5 kHz) exposures used by Bohne (1976). Bohne showed no damage to chinchilla hair cells at the light microscopic level after 9 days of continuous exposure to the 65 dB SPL noise. Others have shown that the guinea pig is slightly less susceptible to noise damage than the chinchilla (Decory et al., 1992). In other studies utilizing chinchillas, a more intense chronic noise exposure (85 dB SPL) resulted in stereocilia damage (Boettcher et al., 1992; Subramaniam et al., 1994). ATP receptors have been proposed to be located near the stereocilia on the apical surface membrane (Housley et al., 1992; Mockett et al., 1994). Thus the decreased response to ATP may be due to some physical or chemical alteration in the ATP receptors located near or on damaged stereocilia. On the other hand, the increased response observed in long OHCs argues against a physical damage and suggests a more complex mechanism.

Aside from damage, a subject's acute and chronic history of sound exposure changes the response of the cochlea to sound. Acute, low-level sound alters the mechanics of the cochlear partition in a complex manner as monitored by quadratic DPOAEs (Kujawa et al., 1995). Chronic, higher level exposure decreases the effect of subsequent noise exposure (Clark et al., 1987; Sinex et al., 1987; Canlon et al., 1988,1992; Campo et al., 1991; Franklin et al., 1991; Boettcher et al., 1992; Mensh et al., 1993; Subramaniam et al., 1994). During this latter effect which is called 'toughening', changes in the mechanics of the cochlea as monitored with DPOAEs have been reported (Franklin et al., 1991; Mensh et al., 1993; Subramaniam et al., 1994). In both these acute and chronic phenomena, the changes in DPOAEs and cochlear mechanics may indicate alterations in the function of the OHCs, possibly involving ATP. Whether the exposure utilized in the present study alters DPOAEs remains to be determined.

In summary, results indicate that the response to ATP was altered in OHCs obtained from noise exposed guinea pigs. We speculate that this is due to an alteration in the number of ATP receptor proteins. Additional research will be necessary to determine whether this mechanism actually underlies the observed phenomena.

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HEARING RESEARCH

Time-varying alterations in the f_2–f_1 DPOAE response to continuous primary stimulation I: Response characterization and contribution of the olivocochlear efferents

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Abstract

The f_2–f_1 distortion product otoacoustic emission (DPOAE) can be observed to undergo gradual alterations in amplitude during continuous ipsilateral stimulation with primary tones. In the present experiments, we characterized the dependence of these amplitude alterations on several stimulus variables (intensity, duration, frequency) and on DPOAE type (quadratic vs cubic) and tested the hypothesis that such alterations are mediated by the olivocochlear (OC) efferents. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles before and after intracochlear application of antagonists (curare, 1 μM; bicuculline, 10 μM; tetrodotoxin, 1 μM) or before and after OC efferent section at the midline of the floor of the IVth ventricle. We confirm previous reports of continuous stimulation-related alterations in the amplitude of the quadratic distortion product, f_2–f_1, and report a novel, suppressive 'off-effect' apparent in f_2–f_1 amplitude following a short rest from such stimulation. Response alterations were sensitive to primary intensity and to duration of rest from continuous stimulation, but were not clearly frequency-dependent over the ranges tested. Corresponding alterations in the amplitude of the cubic nonlinearity, $2f_1$–f_2 were very small or absent. Amplitude alterations in f_2–f_1 were reduced but not blocked by OC efferent antagonists (curare, bicuculline) and were largely unaffected by TTX or by midline brainstem section. All of these manipulations, however, prevented completely the known efferent-mediated contralateral sound suppression of both f_2–f_1 and $2f_1$–f_2 DPOAEs. Taken together, these results do not provide support for efferent control of the f_2–f_1 amplitude alterations observed during continuous ipsilateral stimulation.

Keywords: Olivocochlear efferents; Otoacoustic emissions; Outer hair cells; Quadratic nonlinearity; Cubic nonlinearity

1. Introduction

The quadratic nonlinearity, f_2–f_1, measured as a distortion product otoacoustic emission (DPOAE), displays time-varying amplitude alterations during continuous, primary tone stimulation. Brown (1988) first described a short period of amplitude growth followed by large (often exceeding 15 dB) reductions in f_2–f_1 DPOAE amplitude during continuous low- to moderate-level primary stimulation. Similar amplitude alterations were not observed in the cubic nonlinearity corresponding to the frequency $2f_1$–f_2. In a subsequent investigation, Whitehead et al. (1991) also observed f_2–f_1 but not $2f_1$–f_2 to undergo an initial amplitude increase followed by a gradual decline during continuous primary stimulation. Rarely exceeding 3 dB, the magnitude of the f_2–f_1 amplitude reduction was much smaller than that observed by Brown. Kirk and Johnstone (1993) confirmed the perstimulatory reductions in f_2–f_1 amplitude and reported effects to be most robust within the primary frequency range 2–7 kHz. Again, corresponding changes in the $2f_1$–f_2 distortion product were minimal or absent at any of the frequency combinations tested.

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In contrast to the different behavior of these distortion components during ipsilateral stimulation, both f_2-f_1 and $2f_1-f_2$ distortion products can be altered during presentation of tones or noise to the contralateral ear (e.g., Kirk and Johnstone, 1993; Kujawa et al., 1993, Kujawa et al., 1994; Puel and Rebillard, 1990). When appropriate measures are taken to avoid transcranial crossover of the contralateral signal or middle ear muscle activation, these contralateral influences on ipsilateral DPOAEs reveal clearly an efferent influence on the cochlear mechanics. This efferent control is thought to be mediated primarily by uncrossed medial olivocochlear (UMOC) neurons which synapse directly with outer hair cells (OHCs) and which, due to the predominantly crossed nature of the afferent pathways, are activated primarily by contralateral sound (Warren and Liberman, 1989). Such response suppression can be mimicked by electrical activation of OC neurons (Kirk and Johnstone, 1993; Mountain, 1980; Siegel and Kim, 1982). It can be prevented by cuts to the brainstem that remove this efferent input (Puel and Rebillard, 1990; Puria et al., 1992) and it can be blocked pharmacologically by antagonists of OC efferent activity (Kujawa et al., 1993; Kujawa et al., 1994).

The mechanisms underlying the f_2-f_1 amplitude alterations during continuous ipsilateral stimulation are unknown. Brown (1988) and Kirk and Johnstone (1993) have presented results suggesting that the ipsilateral effects, too, are under efferent control. In the Brown (1988) experiments, f_2-f_1 response alterations were absent or reduced in magnitude in deeply-anesthetized animals. Additionally, they were altered by the ipsilateral presentation of 'novel' stimuli and by stimulation of the contralateral ear during periods of rest from ipsilateral stimulation. In the Kirk and Johnstone (1993) studies, the amplitude alterations were prevented in some animals by intracochlear application of bicuculline, an antagonist of GABA, a putative transmitter of apical cochlear efferents (see Eybalin, 1993 for review) and by tetrodotoxin (TTX). The amplitude alterations were not, however, blocked by strychnine, which has potently antagonized every known OC efferent-mediated effect identified to date (e.g., Bobbin and Konishi, 1974; Desmedt and Monaco, 1962; Kujawa et al., 1994). Yet, in these same animals, strychnine blocked contralateral suppression of f_2-f_1 and abolished the suppressive effects of electrical OC stimulation on both the compound action potential (CAP) of the auditory nerve and the f_2-f_1 DPOAE. Moreover, Whitehead et al. (1991) found no support for efferent control of these amplitude alterations in rabbit. Specifically, responses were not different in awake and anesthetized animals, and they were not altered by contralateral sound. Finally, none of these investigations have revealed corresponding alterations in the amplitude of the $2f_1-f_2$ DPOAE. Given the similar behavior of the f_2-f_1 and $2f_1-f_2$ DPOAEs in response to efferent activation by contralateral sound, it remains unclear why ipsilateral sound-activation of efferent neurons should affect f_2-f_1 and not $2f_1-f_2$ DPOAEs.

Our ultimate goal in these experiments is to identify the mechanism(s) underlying the f_2-f_1 DPOAE amplitude alterations observed during continuous primary stimulation. In this paper, we confirm the amplitude alterations observed by others and report, in addition, a suppressive 'off-effect' of continuous stimulation not described in previous reports. We describe the dependence of these amplitude alterations on several stimulus variables (level, duration and frequency) and on DPOAE type (quadratic vs cubic). In addition, we present results related to our tests of the hypothesis that the amplitude alterations are under efferent control. Toward this end, we studied two antagonists of OC efferent activity (curare, bicuculline) that we have found previously to block contralateral sound suppression of the $2f_1-f_2$ DPOAE (Kujawa et al., 1994), an antagonist of all action potential-mediated activity (TTX) and OC nerve section for their effects on these time-varying changes in f_2-f_1 amplitude. Contralateral suppression of f_2-f_1 and $2f_1-f_2$ DPOAEs was employed as an internal, known efferent-mediated control for the effects of these experimental manipulations.

2. Methods

2.1. Subjects

Pigmented guinea pigs (250–400 g) of either sex were anesthetized (urethane, 1.5 g/kg, i.p.) and tracheotomized but were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment, and temperature was maintained at 38° ± 1° C. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described previously (Kujawa et al., 1994). Briefly, cartilaginous ear canals were exposed and partially removed to allow optimum coupling of the sound delivery systems to the two ears. A subgroup of these animals was used in the cochlear perfusion experiments described in this report. Thus, in all animals, the right auditory bulla was exposed and opened ventrally to gain access to the cochlea and tendons of the right middle ear muscles were sectioned.

2.2. Stimulus generation and response monitoring

The DPOAE under primary investigation during continuous ipsilateral stimulation was the f_2-f_1 DPOAE at 1.25 kHz. For certain ipsilateral and all contralateral stimulus conditions, we also monitored the cubic distortion product at the $2f_1-f_2$ frequency (5

kHz). The instrumentation employed in these experiments has been described (Kujawa et al., 1994). Briefly, responses were elicited by equilevel primary stimuli ($f_1 = 6.25$ kHz; $f_2 = 7.5$ kHz) generated by oscillators, routed through attenuators to separate speakers and delivered to the right ear of each animal by an acoustic probe assembly. Output from the probe microphone was led, via a microphone preamplifier, to a dynamic signal analyzer for averaging (10 discrete spectra) and display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated with these display windows averaged approximately −15 dB SPL for the f_2–f_1 DPOAE and −18 dB SPL for the $2f_1$–f_2 DPOAE when measured at points ±50 Hz of the distortion product frequency. In some experiments, distortion products (f_2–f_1 and $2f_1$–f_2) to other primary pairs were generated; details pertaining to those stimulus conditions will be discussed where appropriate. For contralateral suppression measures, the contralateral stimulus was a 70 dB SPL overall level wideband noise (WBN; 0.9–15.7 kHz), generated and delivered to the left ear as detailed in previous reports (Kujawa et al., 1993; Kujawa et al., 1994).

2.3. Baseline measures

Baseline measures of f_2–f_1 (1.25 kHz) and $2f_1$–f_2 (5 kHz) distortion product growth with increasing stimulus level (25–70 dB SPL in 5 dB steps) were obtained at the right ear of each animal following surgical exposure of the cochlea and middle ear muscle section. Only those animals whose DPOAE growth functions conformed to laboratory norms following these procedures participated in the experiments that followed. A 15 min period without stimulation separated these measures from the next period of primary stimulation.

The influence of continuous, moderate-level (60 dB SPL) primary stimulation on the f_2–f_1 DPOAE at 1.25 kHz was studied in all animals ($N = 35$). The following stimulation and response monitoring protocol was employed: 100 consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time approximately 200 s or 2.3 min of stimulation). Here again, and for all subsequent stimulus manipulations, a 15 min period of rest from primary stimulation separated each test condition from the next.

Contralateral suppression of both the f_2–f_1 and $2f_1$–f_2 DPOAEs also was studied in all animals. Methods were similar to those employed previously (Kujawa et al., 1994). Briefly, 5 consecutive, 10-spectra averages of DPOAE amplitude (to equilevel primaries at 60 dB SPL) were obtained in the absence of contralateral stimulation. The WBN was then introduced to the contralateral ear and 5 consecutive, 10-spectra averages of distortion product amplitude were obtained during contralateral stimulation. The noise was removed and 5 additional 10-spectra averages of DPOAE amplitude were obtained.

Following these baseline response characterizations, animals were employed in experiments designed to characterize the dependence of these amplitude alterations on the stimulus variables of intensity, duration and frequency and on DPOAE type and/or they served as subjects in the cochlear perfusion or nerve section studies.

2.4. Response characterization experiments

Intensity effects

To investigate the influence of primary level on f_2–f_1 DPOAE amplitude alterations, a subgroup of animals was tested with equilevel primaries at 40, 50 and 70 as well as 60 dB SPL. Responses were monitored as described above.

Duration effects

To clarify the dependence of these time-varying changes in f_2–f_1 DPOAE amplitude on continuous primary stimulation, f_2–f_1 amplitude changes associated with intermittent periods of short-duration (5 s) primary stimulation ($L_1 = L_2 = 50$–70 dB SPL) were studied over the same time period. For these comparisons, single 10-spectra averages were obtained at times corresponding to the first average of the continuous series, the 100th average and the final (40th) average following the return to stimulation. Alternatively, following the initial period of continuous primary stimulation (500 s) and 1 min rest, f_2–f_1 distortion product recovery was tracked using 5 s periods of stimulation obtained at 30 s intervals. Results were compared to those obtained in the same animals using our standard (baseline) protocol.

The influence of rest period duration on amplitude changes in the f_2–f_1 DPOAE as recorded following the first period of continuous stimulation was investigated using primaries at 60 dB SPL. This was accomplished by halving (30 s) or doubling (2 min) the period of rest from continuous primary stimulation and then comparing results to those obtained in the same animals with the standard (1 min) rest.

Frequency effects

The influence of primary frequency was investigated by comparing alterations in the amplitude of the f_2–f_1 DPOAE when generated by lower ($f_1 = 2.5$ kHz; $f_2 = 3$

kHz) and by higher (f_1 = 10 kHz; f_2 = 12 kHz) frequency primaries to results obtained with the standard primary pair (f_1 = 6.25 kHz; f_2 = 7.5 kHz). For these measurements, the f_2/f_1 ratio was held constant at 1.2 and primaries were equal in level at 60 dB SPL.

DPOAE type

The effect of DPOAE type on the magnitude and time course of the amplitude alterations during continuous primary stimulation was investigated under several separate conditions: First, amplitude alterations in the cubic ($2f_1-f_2$) DPOAE at 5 kHz were compared to those recorded for the quadratic (f_2-f_1) DPOAE at 1.25 kHz for identical stimulus frequency and intensity conditions (f_1 = 6.25 kHz; f_2 = 7.5 kHz; $L_1 = L_2$ = 60 dB SPL). Second, a $2f_1-f_2$ DPOAE was elicited at 1.25 kHz (f_1 = 1.55 kHz; f_2 = 1.86 kHz; $L_1 = L_2$ = 60 dB SPL) and amplitude alterations observed for this distortion product were compared to those for the f_2-f_1 DPOAE at the same frequency and for the same primary levels. Finally, in an attempt to compare f_2-f_1 and $2f_1-f_2$ DPOAEs similar in baseline amplitude, the $2f_1-f_2$ DPOAE at 5 kHz was elicited by equilevel primaries at 50 dB SPL and response alterations to continuous stimulation under these conditions were compared to those obtained for the f_2-f_1 DPOAE response to higher level (60 dB SPL) primaries.

2.5. Cochlear perfusion experiments

Perfusion experiments were undertaken using methods described previously (Kujawa et al., 1994). The artificial perilymph had a composition of (in mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; NaH_2PO_4, 1; $MgCl_2$, 1; glucose, 11; $NaHCO_3$, 12 with a resulting pH of 7.4 when brought into solution in a deionized and filtered water base. Experimental drugs tested for their effects on ipsilateral and contralateral stimulation-related DPOAE amplitude alterations were the following: bicuculline (bicuculline methiodide, 10 μM, Sigma), curare (d-tubocurarine chloride, 1 μM, Sigma) and TTX (tetrodotoxin, 1 μM, Sigma). The agents were dissolved in the artificial perilymph (pH 7.4). Perfusates were introduced into the cochlear perilymph at a rate of 2.5 μl/min for 10 min through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks.

For the purposes of the perfusion experiments, animals were divided into 4 groups: One group of animals (N = 5) received 8 consecutive perfusions of the control (artificial perilymph) solution alone. Effects of the OC efferent antagonists, bicuculline and curare, were tested in another group of animals (N = 5). These animals first received 2 perfusions of the control (artificial perilymph) solution alone. These perfusions were followed, in order, by a single perfusion of bicuculline, 2 artificial perilymph washes, a single perfusion of curare and two additional artificial perilymph washes. To insure that drugs were not being diluted with replacement CSF during the long response monitoring periods post-perfusion, a third group of animals (N = 4) received these drugs after first undergoing blockade of the cochlear aqueduct as described by Jenison et al. (1985). Effects of TTX were studied in a fourth group of animals (N = 6). Again, the first 2 perfusions were of artificial perilymph alone. These perfusions were followed by a single perfusion of TTX followed by 2 artificial perilymph washes. Immediately following each perfusion, measures of f_2-f_1 and $2f_1-f_2$ DPOAE amplitudes before, during and after contralateral WBN stimulation were obtained followed by measures of f_2-f_1 amplitude during continuous ipsilateral stimulation (60 dB SPL) as described for baseline characterizations.

2.6. OC nerve section experiments

The effect of OC section at the brainstem midline on the ipsilateral stimulation-related changes in f_2-f_1 amplitude was studied in N = 4 animals. Such a section should effectively remove input from the vast majority of ipsilaterally-responsive (i.e., crossed) MOC neurons to each cochlea (Liberman and Brown, 1986). In these animals, the middle cerebellar vermis was aspirated, revealing the floor of the IVth ventricle. Measures of ipsilateral stimulation-related effects on f_2-f_1 DPOAE amplitude to 60 dB SPL primaries were obtained to identify changes in response characteristics that might be associated with these surgical manipulations. A midline cut spanning the anterior-posterior extent of the exposed IVth ventricle floor at a depth of approximately 2 mm was then made and ipsilateral and contralateral stimulation measures were obtained. Results of both sets of measures were compared to baseline data for these animals.

Effects of treatments on ipsilateral and contralateral stimulation-related changes in DPOAE amplitude were quantified using repeated measures analysis of variance (ANOVA) and Tukey post-hoc tests. The care and use of the animals reported on in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

3. Results

3.1. Amplitude alterations during continuous ipsilateral stimulation

During continuous stimulation with moderate-level (60 dB SPL) primaries, the f_2-f_1 DPOAE was ob-

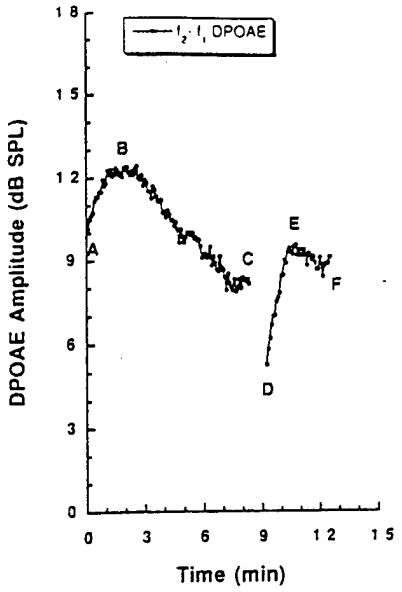

Fig. 1. Effect of continuous primary stimulation on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Each data point represents a 10 spectra average and required 5 s to complete. Break in response amplitude trace (C–D) represents 1 min with no primary stimulation. Points A–F thus identified in each trace were used to calculate magnitudes of component amplitude changes, slopes of suppression and recovery functions and for statistical analyses across animals.

served to undergo stereotyped amplitude alterations. A representative example is shown in Fig. 1. Following a short period of amplitude growth during the first 2 min of continuous primary stimulation, the f_2-f_1 DPOAE underwent a slow decline which either progressed at a similar rate throughout the remainder of the stimulation period (approximately 6–7 min) as in this example, or which gradually fell to a new, lower level and then changed comparatively little during the last few minutes of stimulation. Following a 1 min rest from continuous primary stimulation, a return to stimulation found the DPOAE further suppressed from its pre-rest amplitude. Thereafter, f_2-f_1 amplitude increased rapidly, again reaching a maximum approximately 2 min into the period of continuous primary stimulation. Periodic checks of primary amplitudes revealed changes less than ± 0.1 dB across similar periods of stimulation.

This general response configuration was obtained for 33 of 35 animals tested in this series. In the remaining 2 animals, the post-rest amplitude suppression was absent, but all other amplitude alterations followed the characteristic pattern. Six points (A–F) were identified on each response amplitude function (see Fig. 1). These values were used to calculate the magnitudes of the various amplitude changes, the overall slopes of the suppression and recovery functions and for statistical analyses. The period of amplitude growth observed following stimulus onset will be referred to as an 'on-effect' of continuous primary stimulation. The first such on-effect is represented as the change in amplitude from point A (DPOAE amplitude at the first 10 spectra average) to point B (the 10 spectra average yielding the maximum DPOAE amplitude during the first period of continuous stimulation). In the 35 animals tested with equilevel primaries at 60 dB SPL, this amplitude increase averaged 1.52 ± 0.14 dB at its peak (B), which occurred 1 min 44 s ± 6 s into the period of continuous primary stimulation (A to B slope = 0.88 dB/min). After reaching this amplitude maximum, the response declined slowly during the remainder of the stimulation period (B to C slope = -0.42 dB/min). At the end of this period of continuous primary stimulation (C), the amplitude of the distortion product was reduced 1.24 ± 0.31 dB from its amplitude at onset (A). However, it was reduced from its peak value (B) by 2.75 ± 0.25 dB. At the return to stimulation (D) following a 1 min rest, the f_2-f_1 DPOAE was further suppressed from this pre-rest value (C) by 1.26 ± 0.12 dB. Of interest, the magnitude of the amplitude decline occurring during this 1 min rest from continuous stimulation is 3 times that observed for a comparable time period during which the primaries were delivered continuously. This post-rest amplitude suppression (the change in DPOAE amplitude from point C to point D) will be referred to as the 'off-effect'. Following the return to stimulation, response amplitude increased rapidly (2.44 ± 0.18 dB at point E). This second on-effect (ΔD–E) demonstrated a time course similar to the first, reaching a new maximum 2 min 2 s ± 7 s into the second period of continuous stimulation (D to E slope = 1.20 dB/min). After reaching this amplitude maximum, the response again began on a downward course, clearly evident at point F (E to F slope = -0.43 dB/min). All amplitude changes from baseline reached significance ($P < 0.01$).

When monitored over repeated trials in the same animal, these characteristic f_2-f_1 amplitude alterations demonstrated little variability. Fig. 2 displays the results of repeated averages separated by periods of

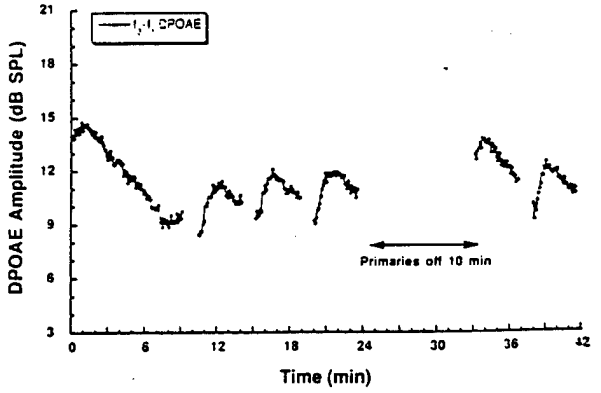

Fig. 2. Effect of repeated primary stimulations separated by 1 min or 10 min rest periods on f_2-f_1 DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL).

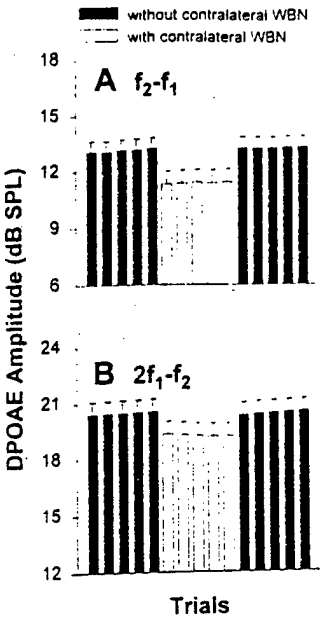

Fig. 3. Effect of contralateral WBN on (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). DPOAEs were monitored before (5 trials, solid bars), during (5 trials, open bars) and after (5 trials, solid bars) presentation of WBN (70 dB SPL) to the contralateral ear. Each 'trial' represents a 10 spectra average and required 5 sec to complete. Data are represented as means ± S.E. (N = 35).

rest (1 min; 10 min) for one representative animal. Although the extent of the amplitude decline is limited in subsequent trials (due to the shortened periods of continuous stimulation; approximately 3.3 vs 8.3 min), on- and off-effects are nearly identical in magnitude and time course and the slopes of the growth and decay portions of the curves remain constant. After a longer period of rest from continuous stimulation (10 min), some additional recovery of distortion product amplitude is evident. Similar results were obtained in 4 additional animals.

3.2. Amplitude alterations during contralateral WBN stimulation

Contralateral suppression of both f_2-f_1 and $2f_1-f_2$ DPOAEs was observed in 34 of 35 animals tested. The f_2-f_1 DPOAE was reduced approximately 1.7 dB (Fig. 3A) and the $2f_1-f_2$ DPOAE was reduced approximately 1.2 dB (Fig. 3B) in the presence of a 70 dB SPL contralaterally-presented WBN. In both cases, the contralateral stimulation-associated reductions in DPOAE amplitude reached significance ($P < 0.001$). When contralateral sound effects are expressed in terms of percent reduction of baseline distortion product amplitude, the f_2-f_1 DPOAE was reduced by roughly 13%, the $2f_1-f_2$ DPOAE by 6%.

3.3. Effects of stimulus variables and DPOAE type

Intensity effects

On average ($N = 9$), the magnitudes of the component amplitude alterations associated with continuous ipsilateral stimulation decreased as stimulus intensity was increased (Figs. 4A–C). Intensity-related changes in on- (ΔA–B; ΔD–E) and off-effect magnitudes (ΔC–D) reached overall significance, with post-hoc comparisons (Tukey) revealing significant differences between values obtained at 50 and 60 dB SPL ($P < 0.01$) and 50 and 70 dB SPL ($P < 0.01$) but not 60 and 70 dB SPL ($P > 0.05$). The magnitude of the pre-rest amplitude decline (ΔB–C) associated with 50 dB SPL primaries was more variable between animals. Thus, intensity-related alterations in the magnitude of pre-rest amplitude decline failed to reach overall significance ($P > 0.05$). Responses to primary stimuli at 40 dB SPL ($N = 3$ animals) were very small and were more variable in amplitude over repeated averages within animals. It could not, therefore, reliably be determined whether effects of continuous stimulation continued to grow as primary level was reduced further.

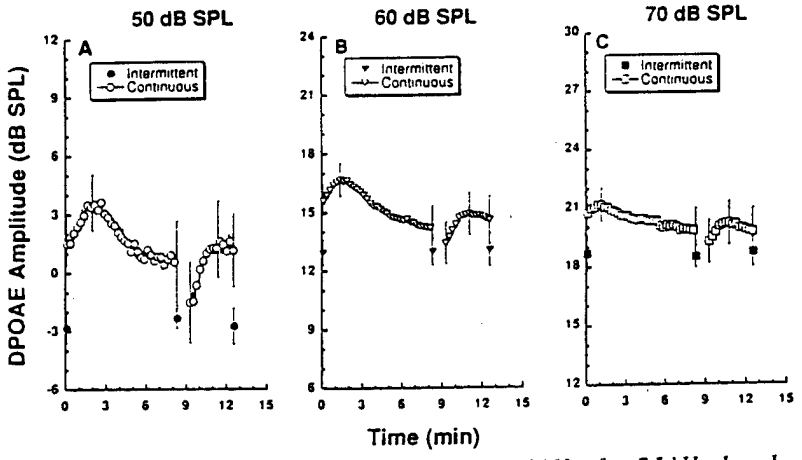

Fig. 4. Effect of primary intensity on f_2-f_1 DPOAE amplitude alterations ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 50$–70 dB SPL) associated with continuous (N = 9) and intermittent (N = 6) stimulations. Data are represented as means ± S.E.

Duration effects

In a subgroup of these animals ($N = 6$), DPOAE amplitudes to 50, 60 or 70 dB SPL primaries presented intermittently (at times corresponding to points A, C and F) remained very stable (Figs. 4A–C). On average, response amplitude to 60 dB SPL primaries varied less than 0.1 dB over a corresponding time period. We did not study how closely-spaced in time the periods of primary stimulation must be in order to produce these characteristic amplitude alterations. However, for periods of intermittent primary stimulation separated by 30 s intervals, distortion product recovery from the post-rest suppression followed a gradual course over the entire period of post-rest monitoring and the degree of amplitude recovery was more variable than that observed using the standard, continuous stimulation protocol.

Both the magnitude of the off-effect and the time course of the subsequent recovery were sensitive to the duration of rest from continuous stimulation. In general, the magnitude of the off-effect associated with either 30 s or 2 min periods of rest was < 0.5 dB. Moreover, although the times to maximum amplitude following the 1 and 2 min rests were similar, this time course was variable across animals following the abbreviated (30 s) rest.

Frequency effects

For these experiments, ($N = 5$) two additional frequency pairs were employed: $f_1 = 2.5$ kHz and $f_2 = 3$ kHz which yielded an f_2-f_1 DPOAE at 500 Hz and $f_1 = 10$ kHz and $f_2 = 12$ kHz which yielded an f_2-f_1 DPOAE at 2 kHz. The magnitudes of the amplitude alterations in the resulting f_2-f_1 DPOAEs were then compared to those observed with the standard primary pair ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz; $f_2-f_1 = 1.25$ kHz). No clear relationship to frequency emerged across the primary frequency range tested: In some animals, f_2-f_1 amplitude alterations were similar in magnitude for the 3 primary pairs tested and in some animals effects were larger when distortion products were elicited by mid- or high- but never low-frequency primaries.

DPOAE type

Under identical conditions of stimulation, amplitude alterations in the $2f_1-f_2$ DPOAE at 5 kHz were substantially smaller than those seen for the f_2-f_1 DPOAE at 1.25 kHz. In general, $2f_1-f_2$ amplitude increased slightly (< 1 dB) over the first few minutes of continuous primary stimulation and subsequently maintained this higher amplitude for the remainder of the stimulation period (Fig. 5). In none of these animals did we observe the continuous stimulation-related decline in $2f_1-f_2$ DPOAE amplitude observed for the f_2-f_1 DPOAE. In most animals, a small (approximately 0.5 dB), suppressive off-effect was evident. Our ability to observe amplitude alterations in this distortion product was not improved when stimulus intensity was reduced to 50 dB SPL, nor was it improved when $2f_1-f_2$ frequency was matched with that of the f_2-f_1 DPOAE under primary investigation in these experiments (1.25 kHz). In these latter groups of animals, however, on- and off-effects were occasionally reversed in sign – that is, on-effects appeared as small reductions in the amplitude of the $2f_1-f_2$ DPOAE and off-effects as small increases in response amplitude.

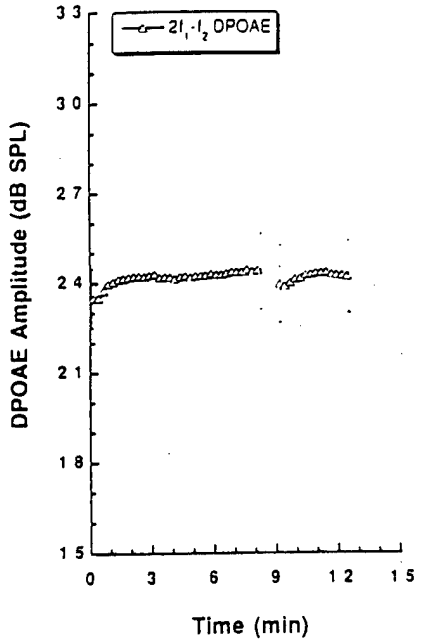

Fig. 5. Effect of continuous primary stimulation on $2f_1-f_2$ DPOAE amplitude ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Data are represented as means ± S.E. across N = 4 animals.

3.4. Pharmacologic blockade of OC efferents

Control perfusions

In contrast to the remarkable stability of the $2f_1-f_2$ DPOAE to the manipulations associated with cochlear perfusion (e.g., Kujawa et al., 1993; Kujawa et al., 1994), the f_2-f_1 DPOAE was altered substantially by perfusion. All components of the perstimulus f_2-f_1 response alterations were enhanced by the first perfusion of the control solution (artificial perilymph; AP). In particular, the pre-rest amplitude suppression (ΔA–C) increased from an average of less than 3 dB pre-perfusion to roughly 8 dB post-AP #1. Similar enhancement of response suppression was not observed when primaries were presented only intermittently over the same time period, suggesting that the additional amplitude decline is not due solely to the long monitoring periods required following perfusion. Response alterations stabilized, however, following the initial AP perfusion and additional control perfusions ($N = 7$) in

each of 5 animals produced no further, significant changes in any response parameter. Thus, for each animal, values obtained following AP perfusion #2 were employed as the new post-perfusion baselines to which drug-related changes were compared. For all post-perfusion measures (control and experimental), contralateral suppression studies preceded immediately our monitoring of ipsilateral stimulation-related changes in f_2–f_1 amplitude. As a result of the prior stimulations, the initial on-effect (ΔA–B) described for the pre-perfusion measures is not observed in these post-perfusion amplitude records. The on-effect represented by the change in f_2–f_1 amplitude from points D–E remains, however, and was employed in statistical analyses of drug effects.

Drug perfusions

Intracochlear perfusion of bicuculline (10 μM) reduced, but did not block the component alterations in f_2–f_1 amplitude during ipsilateral stimulation (Fig. 6A). None of these drug-related changes reached significance ($P > 0.05$). Curare (Fig. 6B), at an order of magnitude lower concentration (1 μM), reduced significantly the pre-rest amplitude decline ($P < 0.05$), but drug-related changes in on- and off-effects failed to reach significance. In contrast, contralateral WBN effects on f_2–f_1 and $2f_1$–f_2 DPOAEs were blocked reversibly by both drugs (Figs. 7A–B), consistent with our previous findings for the $2f_1$–f_2 DPOAE (Kujawa et al., 1994). Also consistent with our previous findings, the amplitude of the $2f_1$–f_2 DPOAE, recorded in the absence of contralateral stimulation, was increased

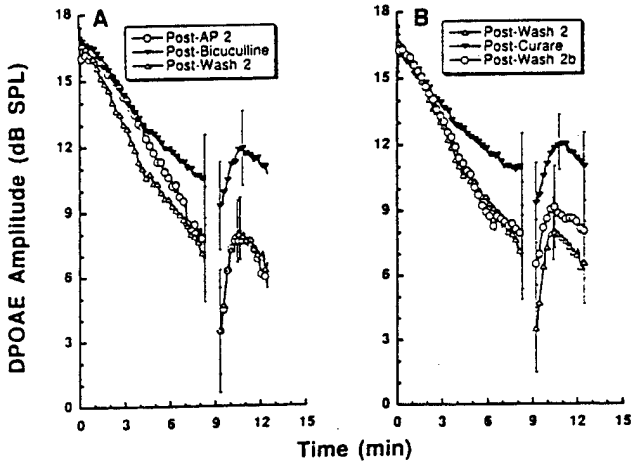

Fig. 6. Effect of OC efferent antagonists on f_2–f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). (A) Response amplitude (means ± S.E.; N = 5) as recorded following the second control perfusion (AP2), bicuculline (10 μM) and the second wash perfusion (W2). (B) Response amplitude (means ± S.E.; N = 5) as recorded following W2 above, curare (1 μM) and two additional wash perfusions (W2b). For reading ease, symbols occur only at every 3rd data point; connecting lines follow all data points.

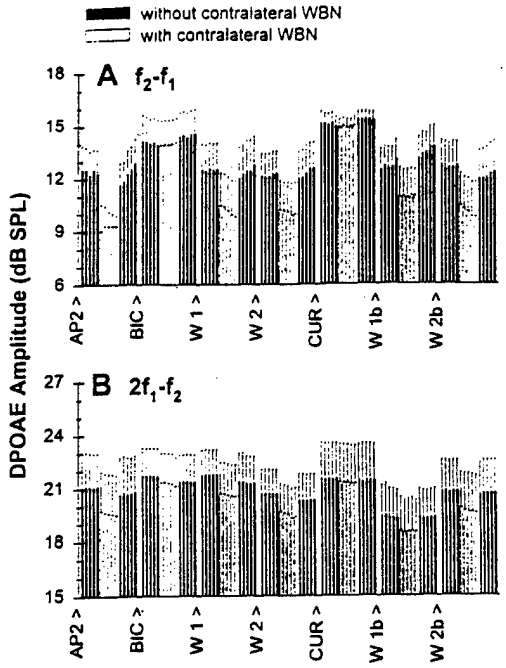

Fig. 7. Effect of OC efferent antagonists on contralateral WBN suppression of (A) f_2–f_1 and (B) $2f_1$–f_2 DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 5) are shown following perfusions of control solutions (AP2), bicuculline (10 μM, BIC), wash perfusions (W1, W2), curare (1 μM, CUR) and two additional washes (W1b, W2b).

from levels observed following perfusions of artificial perilymph. We now extend those observations to include similar effects of these antagonists on f_2–f_1 DPOAE amplitude.

In view of the long post-perfusion monitoring periods employed in these studies, it appeared possible that the inability of the drugs to block the ipsilateral effects might relate to a gradual washing of the drugs from the cochlear perilymph with replacement CSF before post-perfusion measurements could be completed. In pilot experiments, post-drug contralateral suppression was monitored after, rather than before ipsilateral stimulation measures and, in those animals, contralateral suppression could not be observed, suggesting that the drugs remained effective in blocking the efferents even at this extended time post-perfusion. Nevertheless, an additional subgroup of animals ($N = 4$) was tested in which the cochlear aqueduct was blocked prior to perfusion. In these animals, the magnitude of blockade of the ipsilateral effects was not altered substantially from that seen in aqueduct-patent animals. Of interest, when aqueduct-patent animals were subsequently anesthetized with Nembutal (30 mg/kg, i.p.) and ipsilateral stimulation measures repeated, reductions in the magnitudes of the component f_2–f_1 amplitude alterations were similar to those obtained following antagonist perfusions in urethane-anesthetized animals (see Fig. 8).

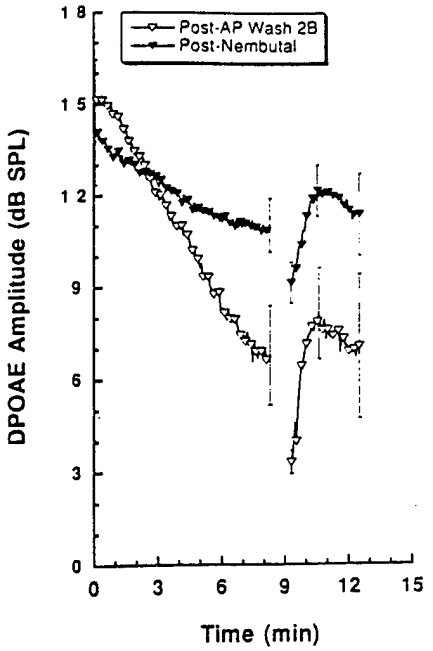

Fig. 8. Effect of Nembutal anesthesia on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz, $L_1 = L_2 = 60$ dB SPL). Perfusion experiments completed under urethane anesthesia (AP Wash 2B) were followed by administration of Nembutal (30 mg/kg, i.p.) to N = 3 animals. Approximately 15 min later, post-Nembutal response measures were obtained. Symbols occur only at every 3rd data point; connecting lines follow all data points.

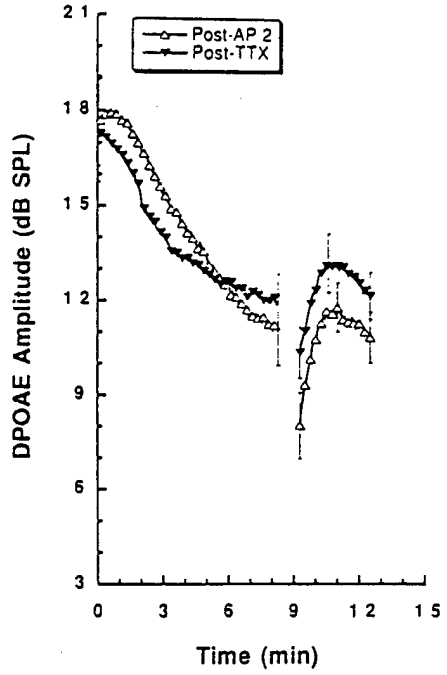

Fig. 9. Effect of TTX on f_2-f_1 DPOAE amplitude alterations during continuous primary stimulation ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz. $L_1 = L_2 = 60$ dB SPL). Shown are response amplitude means ± S.E. (N = 6) following perfusions of control solutions (AP2) and TTX (1 μM). Symbols occur only at every 3rd data point; connecting lines follow all data points.

The concentrations of OC antagonists employed in the perfusion experiments were chosen based on detailed studies of dose-response relations for the pharmacologic blockade of contralateral sound suppression (Kujawa et al., 1994). In those experiments, bicuculline (10 μM) and curare (1 μM) both were effective in blocking that efferent-mediated response. The substantially lesser potency of these drugs in blocking the ipsilateral effects under study here suggested that further clarification of the extent of efferent involvement was necessary. Thus, two additional manipulations were performed in separate groups of animals. In one group ($N = 6$), the cochlear perilymph was perfused with TTX (1 μM) which should block all action potential-mediated activity. Following such perfusions, the magnitude of the off-effect was reduced (Fig. 9), but the pre-rest amplitude decline did not differ significantly from baseline values ($P > 0.05$). In these same animals, contralateral suppression of both distortion products (Figs. 10A–B) was prevented ($P < 0.01$) and the round window-recorded auditory nerve compound action potential (CAP) at 10 kHz was not observed at any intensity (to 102 dB SPL) following perfusion of the cochlear perilymph with TTX. Figs. 10A-B also reveal that the absolute amplitudes of the f_2-f_1 and $2f_1-f_2$ DPOAEs were affected differently by this manipulation: The $2f_1-f_2$ DPOAE is reduced in amplitude

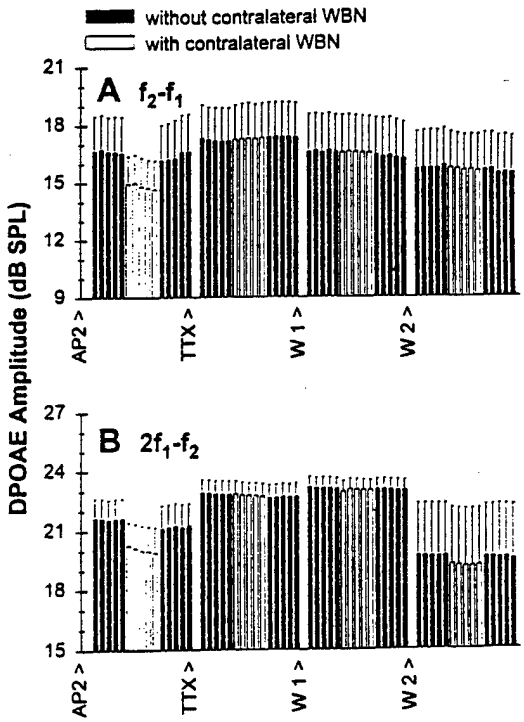

Fig. 10. Effect of TTX on contralateral WBN suppression of (A) f_2-f_1 and (B) $2f_1-f_2$ DPOAEs ($f_1 = 6.25$ kHz, $f_2 = 7.5$ kHz. $L_1 = L_2 = 60$ dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 6) are shown following perfusions of control solutions (AP2), TTX (1 μM) and two wash perfusions (W1, W2).

following attempts at washout; f_2–f_1 amplitude is little changed.

3.5. OC efferent section

A second manipulation performed to clarify the extent of OC involvement in these ipsilateral effects involved section of OC neurons at the midline of the floor of the IVth ventricle. The overall amplitude of the f_2–f_1 DPOAE was reduced following surgical exposure of the floor of the IVth ventricle, but the magnitudes of the component amplitude alterations in this distortion product were essentially unchanged (Fig. 11). Section of the OC efferents at the midline of the IVth ventricle floor produced no further changes in absolute DPOAE amplitude and did not alter substantially the f_2–f_1 amplitude alterations observed during continuous ipsilateral stimulation. In contrast, contralateral suppression of both f_2–f_1 and $2f_1$–f_2 DPOAEs was prevented by midline section (Figs. 12A-B). This may have occurred due to involvement of UMOC neurons in the midline cuts, as these fibers can course very near the brainstem midline, at least in cat (Gifford and Guinan, 1987) and mouse (Brown, 1993b). Here again, f_2–f_1 and $2f_1$–f_2 distortion components

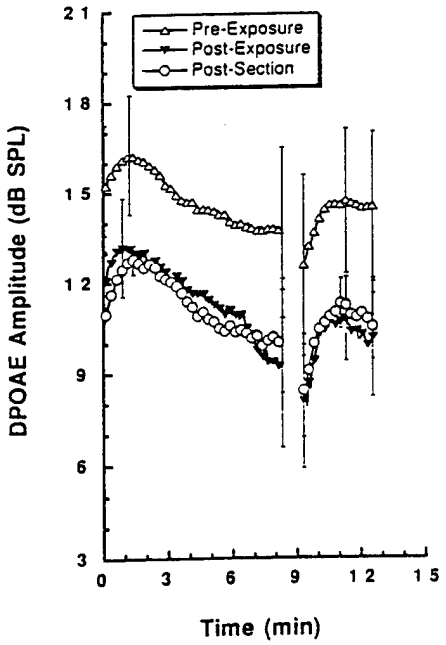

Fig. 11. Effect of OC section on f_2–f_1 DPOAE amplitude alterations during continuous primary stimulation (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). Shown are response amplitude means ± S.E. (N = 4) prior to exposure of the IVth ventricle (Pre-Exposure), following IVth ventricle exposure (Post-Exposure) and following midline section of OC fibers (Post-Section). Symbols occur only at every 3rd data point; connecting lines follow all data points.

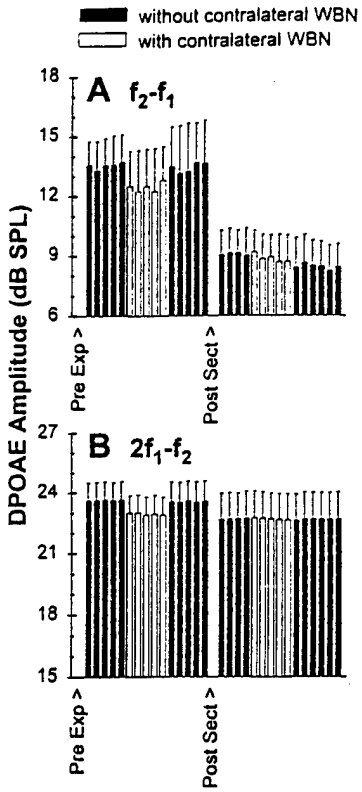

Fig. 12. Effect of OC section on contralateral WBN suppression of (A) f_2–f_1 and (B) $2f_1$–f_2 DPOAEs (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). In each panel, DPOAE amplitudes (means ± S.E., N = 4) are shown before exposure of the IVth ventricle (Pre-Exp) and following midline OC section (Post Section).

display different post-manipulation behaviors: The reduction in absolute distortion product amplitude seen for the f_2–f_1 DPOAE following exposure of the IVth ventricle is not reflected in the $2f_1$–f_2 DPOAE.

4. Discussion

4.1. Alterations in magnitude of auditory system response

Alterations in the magnitude of auditory system response during and following continuous stimulation are well documented. Alterations with short (ms) time courses have been observed in responses of afferent neurons (e.g., Kiang et al., 1965; Young and Sachs, 1973a) and with longer (min) time courses in behavioral responses to sound (e.g., Moore, 1968; Noffsinger and Tillman, 1970; Young and Sachs, 1973b). Response enhancements at the onset of stimulation, perstimulatory declines and post-stimulatory depressions in response magnitude all have been observed. Of importance here, such alterations have been observed for stimulations at intensity levels that should not produce permanent, deleterious effects on cochlear structures.

4.2. f2–f1 amplitude alterations

Given these observations, it should not be surprising that time-varying alterations in the amplitude of the f_2–f_1 DPOAE are observed during continuous primary stimulation. The perstimulatory amplitude changes reported here are qualitatively similar to those reported by others for similar conditions of stimulation. Both Brown (1988) and Whitehead et al. (1991) reported initial increases in f_2–f_1 DPOAE amplitude that were followed by gradual declines during continued primary stimulation. Consistent with results presented by Whitehead and colleagues, we observed the response amplitude maximum (on-effect) to occur approximately 2 min into the period of continuous stimulation. Thereafter, response amplitude declined gradually over the remaining period of stimulation. Although the magnitude of this amplitude decline was similar across animals for 60 dB SPL primaries, it became substantially more variable as primary level was reduced. Brown (1988), employing lower primary levels (20–55 dB SPL), also reported large variations in magnitude of the f_2–f_1 amplitude decline. This variability may account, at least in part, for the reported differences in magnitude of effect between the various investigations of this phenomenon.

A suppressive off-effect of continuous primary stimulation has not been reported by previous investigators of these f_2–f_1 amplitude alterations. In reviewing those reports, it was noted that rest periods from primary stimulation employed in those studies generally exceeded the 1 min rest employed here. Furthermore, when the distortion product was monitored by Brown (1988) after a 3 min rest, partial recovery of response amplitude already was apparent. In the present experiments, the magnitude of this off-effect was substantially reduced when rest periods of either 30 s or 2 min were employed. Thus, it is not surprising that such additional response suppression was not observed by Whitehead et al. (1991) who employed rest periods of 5 min or longer and Brown (1988) who generally employed rest periods exceeding 2–3 min. According to the present results, the lengths of rest used by these investigators would likely have precluded their observation of this time-dependent effect.

4.3. 2f1–f2 amplitude alterations

Consistent with earlier investigations of these perstimulatory DPOAE amplitude changes, corresponding changes in the amplitude of the $2f_1$–f_2 DPOAE were very small or were not observed. In psychophysical paradigms, quadratic (f_2–f_1) and cubic ($2f_1$–f_2) nonlinearities can display different behaviors (Goldstein, 1967; Zwicker, 1979). Moreover, these two distortion components can exhibit different vulnerabilities to cochlear insult (Kujawa and Bobbin, unpublished observations; see also Figs. 10 and 12 in the present report). Such observations suggest that the mechanisms underlying generation of cubic and quadratic cochlear nonlinearities are not identical (for discussion, see Brown, 1993a).

4.4. Contralateral suppression

Although Kirk and Johnstone (1993) failed to observe convincing alterations in $2f_1$–f_2 amplitude during periods of contralateral WBN stimulation, results obtained in the present experiments are in excellent agreement with previous reports of $2f_1$–f_2 amplitude suppression by contralateral noise (Kujawa et al., 1993; Kujawa et al., 1994; Puel and Rebillard, 1990; Puria et al., 1992). Consistent with the report of Kirk and Johnstone, we also observed contralateral sound suppression of the f_2–f_1 DPOAE in virtually all animals tested. On average, the magnitude of this suppression was slightly greater than that observed for the $2f_1$–f_2 DPOAE although, in individual animals, it could be substantially greater.

4.5. Sites and mechanisms underlying response modulation

One possible mechanism that might account for the f_2–f_1 amplitude changes observed during continuous ipsilateral stimulation involves modulation of the cochlear mechanical response by OC neurons. This efferent influence would be accomplished via neurotransmitter (and neuromodulator) substances. Based on the finding of bicuculline (10 μM) blockade of ipsilateral and contralateral stimulation-related alterations in f_2–f_1 amplitude, Kirk and Johnstone (1993) suggested that both processes are controlled via GABAergic efferent pathways. In the present experiments, however, the GABA antagonist, bicuculline, at a concentration of 10 μM, was less effective than the nicotinic cholinergic antagonist, curare, at 1 μM, in antagonizing the perstimulatory amplitude reductions in f_2–f_1. Neither drug blocked these ipsilateral effects completely yet both drugs blocked completely contralateral sound suppression of f_2–f_1 and $2f_1$–f_2 DPOAEs. Our ability to pharmacologically antagonize these ipsilateral effects was not improved substantially in animals in which the cochlear aqueduct had been blocked prior to perfusion. Finally, neither TTX nor OC section altered substantially the ipsilateral stimulation-related effects.

We have previously characterized the pharmacology of contralateral sound suppression of the $2f_1$–f_2 DPOAE response to primaries within the range studied by Kirk and Johnstone (Kujawa et al., 1994). Results of those studies suggested that a nicotinic-like

cholinergic receptor mediated such suppression. Specifically, nicotinic antagonists (α- and κ-bungarotoxins, curare) and strychnine were most potent (IC_{50} values were achieved at nanomolar concentrations of these drugs). Bicuculline also was surprisingly effective in blocking this response suppression ($IC_{50} = 2.39 \times 10^{-6}$). Nicotinic receptor sensitivity to blockade by both strychnine and bicuculline has been demonstrated in several systems (see Kujawa et al., 1994 for review). This sensitivity has been suggested to reflect a strong structural homology between the receptors belonging to the super family that includes receptors for ACh, glycine and GABA (e.g., Grenningloh et al., 1987; Schofield et al., 1987).

At present, we cannot explain the differences between the two investigations with regard to the magnitudes of effect of bicuculline and TTX. The discussion of bicuculline's effects by Kirk and Johnstone (1993), however, suggests that substantial variability between animals in magnitude of drug effect was encountered. Perfusion also increased the variability in the f_2–f_1 DPOAE in our own experiments – both for control and experimental solutions. Additional procedural differences between the investigations may have contributed to observed differences in drug effects, as well. In the present experiments, middle ear muscles were sectioned; in the Kirk and Johnstone studies, animals were paralyzed in an attempt to prevent middle ear muscle contraction. There are differences in anesthetic agents employed and in placement of perfusion and effluent holes in the cochlea between the two investigations.

In view of the lack of effect of TTX and OC section on these response alterations, we also cannot explain the partial blockade of these amplitude alterations by the OC antagonists. However, Nembutal produced a blockade of the ipsilateral effects indistinguishable from that associated with bicuculline and curare. This effect certainly cannot be due to selective or specific blockade of receptors for the OC neurotransmitter. Rather, this finding suggests that, at these concentrations, bicuculline, curare and Nembutal may be acting nonselectively to block a channel operating in the OHCs. Evidence for channel blockade by bicuculline and curare was provided at the level of isolated OHCs by Erostegui et al. (1994).

4.6. Does efferent control underlie f_2–f_1 DPOAE amplitude alterations?

Results of the present experiments do not make it possible to specify the mechanism(s) underlying the perstimulatory alterations in f_2–f_1 amplitude described here. The results suggest, however, that they are not primarily the result of efferent control. Moreover, as reviewed by Young and Sachs (1973a), since sound-induced increases in efferent firing rate are maximum at stimulus onset (Fex, 1962) and OC inhibition of afferent activity decreases with time during a maintained stimulus (Wiederhold and Kiang, 1970), it follows that efferent activation should be associated with a perstimulatory response suppression which is maximum near stimulus onset, rather than the observed enhancement of suppression with time. The experiments cannot rule out an entirely peripheral efferent effect on f_2–f_1 DPOAE amplitude; however, if such an influence exists, it does not appear to involve action potential-mediated activity. Additionally, it would have to follow a time course much longer than that described to date for OC-mediated effects – even the recently-described 'slow effects' of OC stimulation (Sridhar et al., 1994) which demonstrated a pharmacology consistent with that previously described for contralateral suppression (Kujawa et al., 1994). A second mechanism that might underlie these ipsilateral effects could involve local, adaptive changes occurring at the level of the hair cells. Adaptation of the hair cell transduction current during sustained mechanical deflection has been demonstrated in vertebrate (although not yet mammalian) hair cells (e.g., Crawford et al., 1989; Eatock et al., 1987; Assad and Corey, 1992). Further, it has been suggested that distortion product responses arise from nonlinearities in hair cell channel gating mechanisms (Jaramillo et al., 1993). It is possible that such adaptive changes could be reflected in otoacoustic emissions. In previous work by Siegel et al. (1982) temporary reductions in the amplitudes of f_2–f_1 and $2f_1$–f_2 distortion components recorded at the level of single afferent units were observed following stimulations as low as 60 dB SPL. The authors noted that these alterations resembled those observed following higher-level, fatiguing stimulations. In subsequent papers, we will report the results of experiments in which we investigated the influence of local, calcium-dependent mechanisms and chronic, moderately-intense noise exposures on these amplitude alterations.

Acknowledgments

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HEARING RESEARCH

Time-varying alterations in the f_2-f_1 DPOAE response to continuous primary stimulation
II. Influence of local calcium-dependent mechanisms [1]

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Abstract

The distortion product otoacoustic emission (DPOAE) corresponding to the frequency f_2-f_1 displays stereotyped, time-varying amplitude alterations during continuous primary tone stimulation. The origin of these alterations is unknown; however, evidence that efferent neurons contribute little to the changes has been presented (Kujawa et al., 1994●Kujawa et al., 1995; Lowe and Robertson, 1995). The present investigation examines the hypothesis that these alterations in f_2-f_1 amplitude are a reflection of local, Ca^{2+}-dependent mechanisms involving the outer hair cell (OHC) response to sustained stimulation. Experiments were performed using urethane-anesthetized guinea pigs with sectioned middle ear muscles. Intracochlear perfusion was employed to reversibly lower perilymph Ca^{2+} levels and to introduce antagonists and agonists of L-type Ca^{2+} channels. Manipulations that lowered available Ca^{2+} (zero Ca^{2+} artificial perilymph; zero Ca^{2+} with BAPTA) or that blocked its entry into the cell via L-type Ca^{2+} channels (nimodipine) reduced, prevented or reversed the perstimulatory changes in f_2-f_1 DPOAE amplitude. These perilymph manipulations also reduced the overall amplitude of this distortion component while perfusion of an L-type Ca^{2+} channel agonist (Bay K 8644) increased its amplitude. Mg^{2+} did not substitute for Ca^{2+}, suggesting that these are not merely divalent cation effects. Results are consistent with the hypothesis that continuous stimulation-related changes in f_2-f_1 DPOAE amplitude are sensitive to perilymph Ca^{2+} levels and to the function of L-type Ca^{2+} channels. However, nimodipine also reduced the endocochlear potential (EP) and Bay K 8644 increased the EP. The sensitivity of both the perstimulatory changes in f_2-f_1 DPOAE amplitude and the EP to the latter drugs leaves their site(s) of action unresolved.

Keywords: Adaptation; Calcium; Distortion product; Non-linearity; Outer hair cell; Otoacoustic emission

1. Introduction

The f_2-f_1 distortion product otoacoustic emission (DPOAE) displays stereotyped, time-varying amplitude alterations during continous, primary tone stimulation. If monitored after a period of silence, the f_2-f_1 DPOAE response to continuous primaries undergoes a short period of amplitude growth followed by a progressive reduction in amplitude during continued stimulation (Brown, 1988; Whitehead et al., 1991; Kirk and Johnstone, 1993; Kujawa et al., 1994●Kujawa et al., 1995; Lowe and Robertson, 1995). If the primaries are then removed and there is a 1 min rest from such stimulation, further response decrement is apparent immediately upon re-introduction of the primary tones (Kujawa et al., 1994●Kujawa et al., 1995). The response subsequently repeats its initial pattern of amplitude growth followed by slow decline as primary tone stimulation is continued. Similar amplitude alterations have not been observed in the cubic distortion product, $2f_1$-f_2.

The mechanisms underlying these stimulation-related changes in the f_2-f_1 DPOAE remain unclear. Medial olivocochlear (MOC) efferent neurons respond to sound (e.g., Liberman and Brown, 1986) and are anatomically well-situated to influence the response properties of outer hair cells (OHC). Electrical and acoustic activation of MOC neurons alters DPOAEs — effects presumed related to MOC influences on OHCs (Mountain, 1980; Puel and

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Rebillard, 1990). Thus, one possible mechanism that might account for the f_2-f_1 amplitude changes observed during continuous ipsilateral sound stimulation involves modulation of OHC activity by ipsilaterally responsive MOC neurons. However, two investigations that have tested this efferent-control hypothesis by studying the effects of antagonists of cochlear efferent activity (Kujawa et al., 1995) and of midline section of this fiber pathway (Kujawa et al., 1995; Lowe and Robertson, 1995) observed little impact of these manipulations on such changes in f_2-f_1 DPOAE amplitude. Thus, it appears unlikely that the efferent innervation to the cochlea plays a substantial role in producing these particular continuous stimulation-related f_2-f_1 DPOAE amplitude alterations.

A second mechanism that might underlie these effects involves local changes occurring at the level of the hair cells in response to sustained stimulation. Isolated OHCs are capable of responding to applied AC electrical stimulations with rapid, phasic changes in length (e.g., Ashmore, 1987; Brownell et al., 1985). If similar length changes occur in vivo in response to sound, they may allow the OHCs to amplify the mechanical stimulus to the inner hair cells (IHC) by providing a cycle-by-cycle boost at low stimulus levels (see Dallos, 1992 for review). Inherent in such length changes are non-linearities that are revealed as a tonic, or DC, component that follows the envelope of the stimulus waveform (e.g., Santos-Sacchi, 1989; Evans et al., 1991). DC motions also can be observed in isolated OHCs in response to acoustic (mechanical) stimulations (Canlon et al., 1988; Brundin and Russell, 1994) and these motions are reflected in DC position shifts of the reticular lamina (Brundin et al., 1992) and basilar membrane (LePage, 1987). It has been suggested (Zenner and Ernst, 1993) that, in vivo, DC OHC length changes could influence the operating point of the stereocilia and, by changing their sensitivity to displacement, may contribute to such processes as adaptation during sustained stimulation and the temporary changes in sensitivity that follow overstimulation. Unlike the phasic (AC) component of the electromotile response, which is comparatively immune to trauma and does not require immediate metabolic (Ca^{2+}, ATP) support (Kachar et al., 1986; Ashmore, 1987), tonic (DC) motions of OHCs are highly vulnerable to overstimulation (Evans et al., 1991) and to pharmacologic manipulations and are metabolically dependent (Canlon and Brundin, 1991).

The mechanical response of the cochlear partition to sound, at least for lower levels of stimulation, is thought to reflect the physiologically vulnerable, active motion of the OHCs (Robles et al., 1991; Mammano and Ashmore, 1993). DPOAEs are a reflection of non-linearities in that mechanical response (Robles et al., 1991). Although the specific mechanisms underlying distortion product generation are not fully understood, they are widely regarded to directly involve the OHCs. With regard to the acoustic distortion product at f_2-f_1, Brown (1993, 1994) has argued that this non-linearity may be closely tied to the tonic motile response of OHCs to the beat cycle envelope of the two-tone stimulus. According to Brown, if these highly non-linear OHC length changes result in rectification of the response to bitonal stimulation, a difference frequency (f_2-f_1) would be generated which could propagate outward to be recorded as an acoustic distortion product emission. Such responses may provide an indirect means to study, in vivo, the OHC response to sustained stimulation.

We have speculated previously (Kujawa et al., 1994●Kujawa et al., 1995) that the slow changes in f_2-f_1 DPOAE amplitude that are observed during sustained, moderate-level primary stimulation are the result of Ca^{2+}-dependent, adaptive changes occurring at the level of the OHCs. The present investigation was undertaken to examine that hypothesis. Intracochlear perfusion techniques were employed to reversibly lower perilymph Ca^{2+} levels and to introduce antagonists and agonists of L-type Ca^{2+} channels. We studied the effects of these perilymph Ca^{2+} manipulations on the time-varying, perstimulatory changes in f_2-f_1 DPOAE amplitude. Preliminary results have been reported (Kujawa et al., 1994●).

2. Methods

2.1. Subjects

Experiments were performed on pigmented guinea pigs of either sex weighing between 250 and 400 g. Anesthetized animals (urethane, Sigma; 1.5 g/kg, i.p.) were tracheotomized and were allowed to breath unassisted. ECG and rectal temperature were monitored throughout each experiment and rectal temperature was maintained at 38° ± 1°C by a heating pad. Additional urethane was administered as required to maintain an adequate depth of anesthesia.

Surgical procedures have been described (Kujawa et al., 1994●). Briefly, cartilaginous ear canals were exposed and partially removed to allow direct and optimum coupling to the sound delivery system. In all animals the right auditory bulla was exposed using a ventrolateral approach and tendons of the right middle ear muscles were sectioned.

2.2. DPOAE: stimulus generation and response monitoring

DPOAEs (f_2-f_1, 1.25 kHz; $2f_1$-f_2, 5 kHz) were elicited by equilevel primary stimuli (f_1 = 6.25 kHz; f_2 = 7.5 kHz; f_2/f_1 = 1.2) delivered to the right ear of each animal by an acoustic probe assembly (see Kujawa et al., 1995 for additional details). Output from the probe microphone was led via a microphone preamplifier to a dynamic signal analyzer for fast Fourier transform analysis (averaging 10 discrete spectra) and spectral display (span = 1 kHz; CF = DP frequency; BW = 3.75 Hz). The noise floors associated

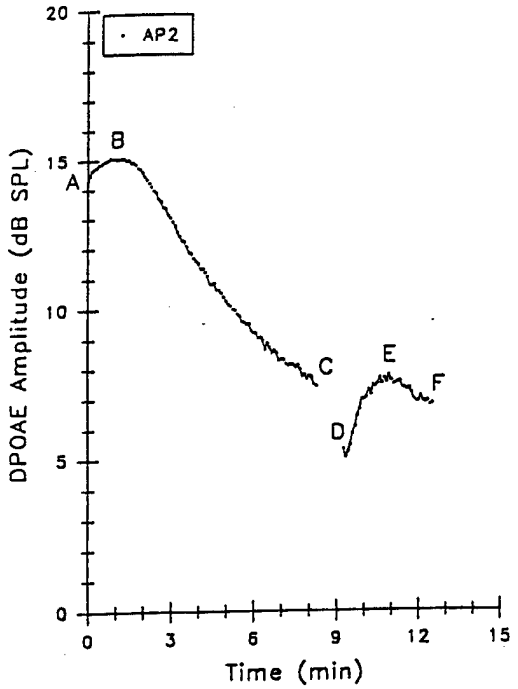

Fig. 1. A typical example of the effect of continuous primary stimulation on f_2-f_1 DPOAE amplitude (f_1 = 6.25 kHz, f_2 = 7.5 kHz, $L_1 = L_2$ = 60 dB SPL). Each data point represents a 10-spectra average and required 5 s to complete. Break in response amplitude trace (C,D) represents 1 min with no primary stimulation. Points A–F thus identified in each trace were used to calculate magnitudes of component amplitude changes for statistical analyses of treatment effects across animals. This particular trace was obtained following a control artificial perilymph perfusion (AP2).

with these display windows averaged approximately −12 dB SPL for the f_2-f_1 DPOAE and −15 dB SPL for the $2f_1$-f_2 DPOAE.

The f_2-f_1 DPOAE response to continuous, moderate-level (60 dB SPL) primaries was monitored as described in a previous report (Kujawa et al., 1995). In brief, 100 consecutive 10-spectra averages of distortion product amplitude were obtained during continuous primary stimulation. Each of these averages required approximately 5 s to complete for a total of 500 s (8.3 min) of stimulation. The primary tones were then simultaneously turned off and there was a 1 min rest from primary stimulation. Following this rest, the primaries were re-introduced and 40 consecutive 10-spectra averages of distortion product amplitude were obtained (total time ≈ 200 s or 2.3 min of stimulation). Six points (A–F) were identified on each response amplitude function (see Fig. 1). These values were used to characterize the component amplitude alterations as detailed elsewhere (Kujawa et al., 1995). They include an 'on-effect' (A,B), a 'slow decline' (B,C), an 'off-effect' (C,D) and a 'second on-effect' (D,E) following the return to stimulation. The distortion products at f_2-f_1 and $2f_1$-f_2 also were studied as growth functions of increasing primary level over the range 20–70 dB SPL in 5 dB steps.

2.3. Cochlear perfusion experiments

Perfusion studies were undertaken using methods described previously (Kujawa et al., 1994●). The 'normal' artificial perilymph had a composition of: 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 11 mM glucose, and 12 mM $NaHCO_3$. An artificial perilymph without added Ca^{2+} ('0 Ca^{2+}' artificial perilymph) was obtained by omitting the $CaCl_2$ and the NaH_2PO_4. This solution also was used to dissolve and deliver the 5 mM BAPTA and the 2 and 4 mM Mg^{2+}. Nimodipine (Research Biochemicals International) and Bay K 8644 (S(−)-Bay K 8644, Research Biochemicals International) were dissolved in dimethylsulfoxide (DMSO, Sigma) and stored in the dark at −20°C for no more than 1 week. The nimodipine in DMSO, Bay K 8644 in DMSO, and DMSO alone were mixed with artificial perilymph on the day of use at desired concentrations (nimodipine: 0.1–10 μM; Bay K 8644: 0.1–10 μM; DMSO: 0.001–0.1%). Otherwise, all perfusion solutions were freshly prepared on the day of use. Perfusates (pH 7.4) were introduced at room temperature into the cochlear perilymph at a rate of 2.5 μl/min for 10 min (0 Ca^{2+}; 0 Ca^{2+} with 2 or 4 mM Mg^{2+}; 0 Ca^{2+} with BAPTA) or 15 min (DMSO; nimodipine in DMSO; Bay K 8644 in DMSO) through a hole in basal turn scala tympani and were allowed to flow from the cochlea through an effluent hole placed in basal turn scala vestibuli. Effluent was absorbed within the bulla using small cotton wicks. In all animals, the first two perfusions were of 'normal' artificial perilymph alone. These perfusions were accomplished to achieve a stable baseline to which effects of subsequent alterations in the artificial perilymph and drugs could be compared. They were followed by perfusions of the altered artificial perilymph ('0 Ca^{2+}' artificial perilymph) or experimental drug. Test solutions were washed from the cochlear perilymph with 'normal' artificial perilymph. A 10–15 min period without stimulation (during perfusions) separated each period of primary stimulation from the next. Following each period of perfusion, we monitored f_2-f_1 amplitude to 60 dB SPL primaries, presented continuously as described. Additionally, growth functions for this distortion component and the 5 kHz $2f_1$-f_2 DPOAE response to the same primary frequencies were obtained over the range 20–70 dB SPL in 5 dB steps. Data were obtained from five animals per treatment.

2.4. Endocochlear potential experiments

In separate groups of animals (n = 5 each), nimodipine, Bay K 8644 and the solvent DMSO were tested for their effects on the endocochlear potential (EP). Methods used to measure this potential have been described (Bobbin et al., 1990). Briefly, the bone over the basal turn scala media

was shaved and a small hole was made through the thinned bone. A glass microelectrode, filled with 150 mM KCl and connected to a DC amplifier (Grass P15), was passed through the hole and inserted into the scala media to record the EP. The output of the amplifier was connected to a digital voltmeter and chart recorder to obtain hard copy records of the EP values.

All perfusions in the EP study were 15 min in duration. The perfusion pump was turned on and the pipette was inserted into the infusion hole. Approximately 10 s later the first EP value was recorded. Additional measurements of the EP were obtained at 1 min intervals for 15 min after which the pipette was removed and filled with the next solution. Approximately 4 min elapsed between perfusions. The 16 values (0–15 min) were then averaged across animals to describe drug effects on the EP during the course of the perfusions.

Effects of treatments were quantified using repeated-measures analysis of variance (ANOVA) procedures and Newman-Keuls post-hoc tests. The care and use of the animals reported on in this study were approved by the university's Animal Care and Use Committee.

3. Results

3.1. Effects of continuous primary stimulation on the f_2-f_1 DPOAE

During continuous, equilevel primary stimulation (60 dB SPL), f_2-f_1 DPOAE amplitude displayed the time-varying changes we have described previously (Kujawa et al., 1995; see also Fig. 1). These amplitude changes include an initial increase in f_2-f_1 level that reached a maximum 1–2 min after the start of stimulation ('on-effect'; A,B), a 'slow-decline' in amplitude that followed with continued stimulation (B,C), the additional amplitude depression observed immediately following a short (1 min) rest from stimulation ('off-effect'; C,D) and the rapid recovery to a second maximum, again roughly 2 min following the return to continuous primary stimulation (second 'on-effect'; D,E). Such amplitude changes were observed during baseline response characterizations in all animals tested.

3.2. Actions of low Ca^{2+}, high Mg^{2+} and BAPTA on DPOAEs

Perfusion of the cochlea with an artificial perilymph solution without added Ca^{2+} (0 Ca^{2+}) had little effect on the component f_2-f_1 amplitude alterations to continuous primary stimulation (Fig. 2A). The most obvious effect was a small ($\approx$ 2 dB) reduction in the overall amplitude of the f_2-f_1 DPOAE response to 60 dB SPL primaries. When compared to values corresponding to points a, c and d (see Fig. 1 for reference) obtained following the second control perfusion (Ap2), this overall amplitude reduction was not significant ($P > 0.05$). Subsequent perfusions of solutions containing no added Ca^{2+} with added Mg^{2+} (2 and 4 mm) resulted in larger amplitude reductions (Fig. 2B), with effects reaching significance ($P < 0.01$) for the 4 mM Mg^{2+} treatment. The magnitudes of the perstimulatory slow decline and 'off-effect' were significantly enhanced ($P < 0.01$) by both 2 and 4 nM Mg^{2+}, with the 4 nM Mg^{2+} solution actually reversing the direction of the

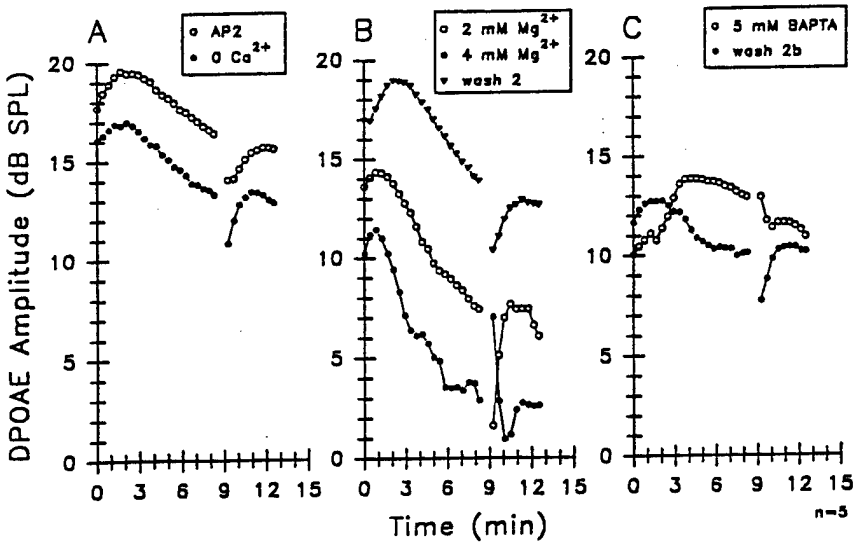

Fig. 2. Effect of altering perilymph Ca^{2+} or Mg^{2+} on perstimulatory changes in f_2-f_1 amplitude. Response amplitude (means: $n = 5$) recorded following successive perfusions in the same animals of: normal artificial perilymph (AP2), artificial perilymph with no added Ca^{2+} (0 Ca^{2+}), 0 Ca^{2+} artificial perilymph with 2 mM Mg^{2+} (2 mM Mg^{2+}) or with 4 mM Mg^{2+} (4 mM Mg^{2+}), a wash perfusion with normal artificial perilymph (wash 2), 0 Ca^{2+} artificial perilymph with 5 mM BAPTA (5 mM BAPTA) and a final wash with normal artificial perilymph (wash 2b). Only every 5th data point is plotted for clearer identification of symbol type. Pooled standard errors were: AP2 = 1.20; 0 Ca^{2+} = 0.75; 2 mM Mg^{2+} = 1.19; 4 mM Mg^{2+} = 2.55; wash 2 = 0.72; 5 mM BAPTA = 1.62 and wash 2b = 1.42 dB. See Fig. 1 legend for additional information.

'off-effect'. In addition, the 'on-effect' maximum (point B) was shifted to progressively earlier points in time following perfusions of the 0 Ca^{2+} and the 0 Ca^{2+} with 2 and 4 nM Mg^{2+} solutions. All actions were readily reversed by perfusions with the 'normal' artifical perilymph (wash 2, Fig. 2B). The Ca^{2+} chelating agent, BAPTA (5 nM; in 0 Ca^{2+} artificial perilymph) was perfused next, and appeared to reverse the directions of all components of the perstimulatory f_2-f_1 DPOAE amplitude changes (Fig. 2C). These actions of BAPTA on the component amplitude alterations were reversed following wash perfusions with normal artificial perilymph (wash 2b) although overall distortion product amplitude did not return completely to pre-drug levels.

Drug-related changes in f_2-f_1 growth functions paralleled the effects observed for this distortion component during continuous primary stimulation (monitored in three of the five animals tested). Perfusion with artificial perilymph solutions without added Ca^{2+} (0 Ca^{2+}, 0 Ca^{2+} with Mg^{2+}) had little effect on low-level portions of the f_2-f_1 growth function (2 ± 4 dB at 40 dB SPL) and reduced f_2-f_1 amplitude at primary levels greater than ≈ 50 dB SPL (e.g., at 65 dB SPL, reductions of 3 ± 1 dB, 7 ± 2 dB and 9 ± 2 dB were obtained for 0 Ca^{2+}, 2 nM Mg^{2+} and 4 nM Mg^{2+} treatments, respectively). The $2f_1$-f_2 DPOAE also was monitored as a function of increasing stimulus level following drug perfusions. This distortion product was essentially unchanged by these perilymph manipulations (shifts averaged 2 dB or less at all stimulus levels). BAPTA in 0 Ca^{2+} artificial perilymph had large effects on both distortion components. Growth functions were shifted to the right, with greater effects seen at lower stimulus levels than at higher levels (e.g., the $2f_1$-f_2 DPOAE response to 40 dB SPL primaries was reduced by 8 ± 1 dB and the response to 65 dB SPL primaries was reduced by 4 ± 1 dB).

3.3. Actions of DMSO, nimodipine and Bay K 8644 on DPOAEs

Perfusion of the solvent (DMSO) alone, in the same increasing concentrations employed in the nimodipine and Bay K 8644 solutions, had little effect on the time-varying alterations in f_2-f_1 amplitude (Fig. 3). The post-DMSO decrease in the starting value of f_2-f_1 DPOAE amplitude (point A) was not significant ($P > 0.05$; Figs. 3 and 4), nor was the increase in the magnitude of the 'slow decline' (B,C). At higher concentrations, the 'off-effect' was progressively reduced; however, this reduction reached significance ($P < 0.05$) only at the highest DMSO concentration

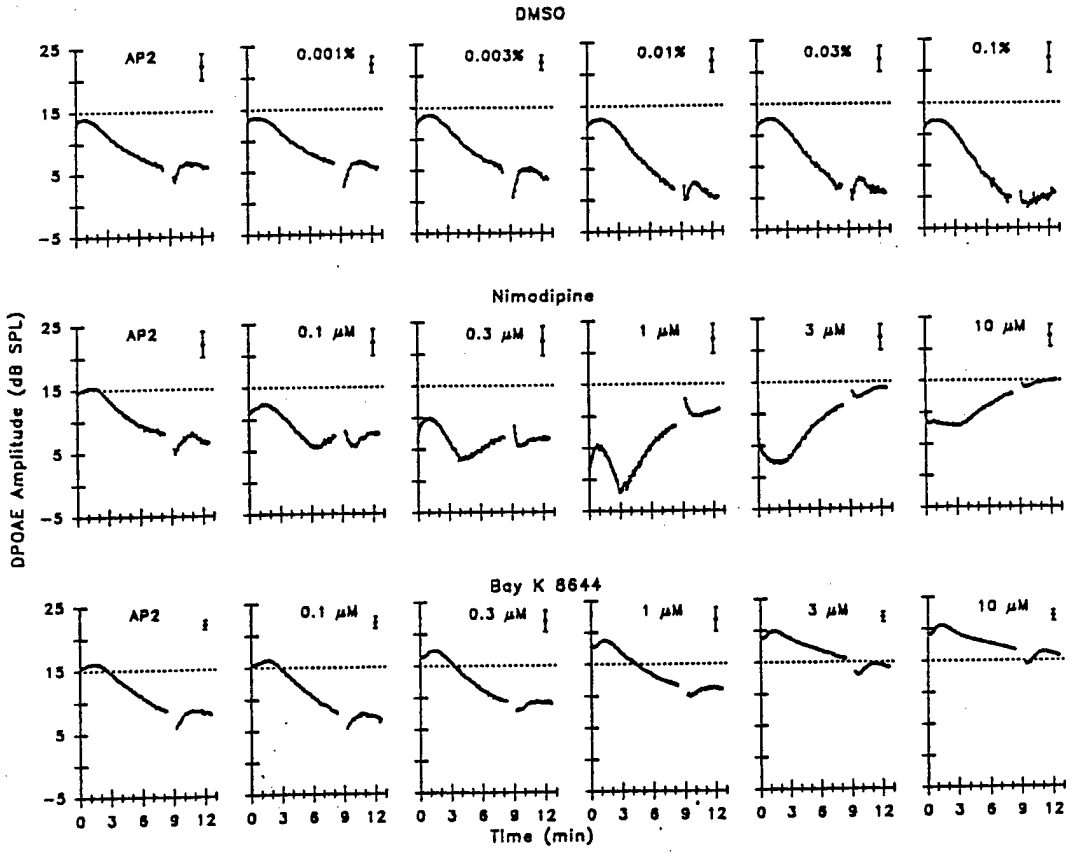

Fig. 3. Effect of increasing concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO ($n = 5$ animals each) on the f_2-f_1 DPOAE response to continuous primaries at 60 dB SPL. Mean response amplitude for every data point is shown. Pooled errors are shown in the upper right hand corner of each frame. The dashed line is drawn at 15 dB for visual reference. See Fig. 1 legend for additional information.

employed (0.1%).

In contrast to the small effects of the solvent alone, the drugs had large effects. The actions of nimodipine were very complex (Figs. 3 and 4). The starting amplitude of the f_2-f_1 DPOAE (A) was decreased by 0.1–1.0 μM nimodipine. At higher concentrations (3 and 10 μM), this value began to return towards pre-drug levels. The 'on-effect' (A,B) was increased by the same low concentrations of nimodipine (0.1–1 μM), whereas higher concentrations (3–10 μM) appeared to reverse the 'on-effect'. It is possible that this reversal may have resulted from the slowly developing reversal of the 'slow decline' (B,C) which is first seen following perfusion of 0.1 μM nimodipine as an increase in f_2-f_1 magnitude approximately 6 min into the recording. With increasing drug concentrations, this amplitude recovery (or reversal of the slow decline) moves earlier in time: 0.1 μM = 6 min; 0.3 μM = 4 min; 1.0 μM = 3 min; 3.0 μM = 2 min and at 10 μM it may even begin at zero min. The 'off-effect' and the 'second on-effect' reversed at the lowest concentration of nimodipine tested (0.1 μM) and remained reversed over the course of the subsequent perfusions with increasing drug concentrations.

Compared to nimodipine, Bay K 8644 appeared to be very straightforward in its actions on the f_2-f_1 DPOAE (Figs. 3 and 4). Bay K 8644 increased, in a dose-related fashion, the absolute amplitude of this distortion component measured at point A. This increase reached significance ($P < 0.05$) for drug concentrations at 1–10 μM (Fig. 4). Bay K 8644 did not substantially alter the 'on-effects' (A,B; D,E) or the 'off-effect' (C,D). On the other hand, it did decrease the slope of the 'slow-decline' (B,C). Interestingly, the overall shapes of the response alterations after 3 and 10 μM nimodipine appear to be the reverse of the overall shapes observed after 3 and 10 μM Bay K 8644 (Fig. 3).

Drug-related changes in f_2-f_1 growth functions again paralleled effects observed during continuous primary stimulation (Figs. 5 and 6). DMSO had very little effect on the growth function, with the exception that it appeared to diminish the 'knee' in the function which occurred at approximately 60 dB SPL (Fig. 5). Nimodipine had complex effects in that low concentrations (0.1–0.3 μM) had little impact, a slightly higher concentration (1 μM) enhanced the function and higher concentrations (3–10 μM) reduced the low-to-moderate-level portions while continuing to enhance the higher-level portions of the growth functions. Bay K 8644 enhanced the growth function at all primary levels — especially for drug concentrations of 3 and 10 μM (Figs. 5 and 6). By comparison, neither DMSO nor Bay K 8644 altered $2f_1$-f_2 growth functions whereas nimodipine reduced them, especially at low levels of stimulation (Figs. 5 and 6).

3.4. Actions on the endocochlear potential

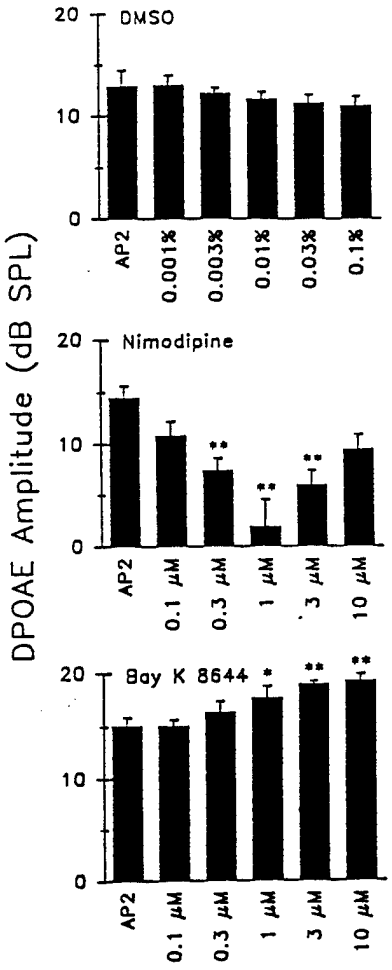

Fig. 4. Effect of increasing concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on the first f_2-f_1 DPOAE amplitude value recorded (point A) following each perfusion. Points that are significantly different from their respective AP2 or DMSO alone concentrations are designated: $^*P < 0.05$; $^{**}P < 0.01$. Data are represented as means ± SE ($n = 5$ animals per drug treatment).

The first perfusion of artificial perilymph increased the EP slightly. The second perfusion of the artificial perilymph (AP2) did not alter the EP further. Thus, as with the DPOAEs, the AP2 value is considered the control for each series of perfusions. DMSO perfusions served as controls for the concentration of DMSO in the drug solutions. For each experiment, the last (15 min) value of the EP recorded during the second control perfusion (AP2) was subtracted from the corresponding EP value recorded during perfusion of each concentration of drug. Dose–response curves of drug effects on the EP (means ± SE) were constructed from the resulting difference values and are displayed in Fig. 7.

Perfusions of increasing concentrations of the drug solvent, DMSO, had no significant effect ($P > 0.05$) on the EP when compared to its AP2 (Fig. 7). The experimental drug nimodipine produced a reduction of the EP that was significantly different ($P < 0.05$) from effects associ-

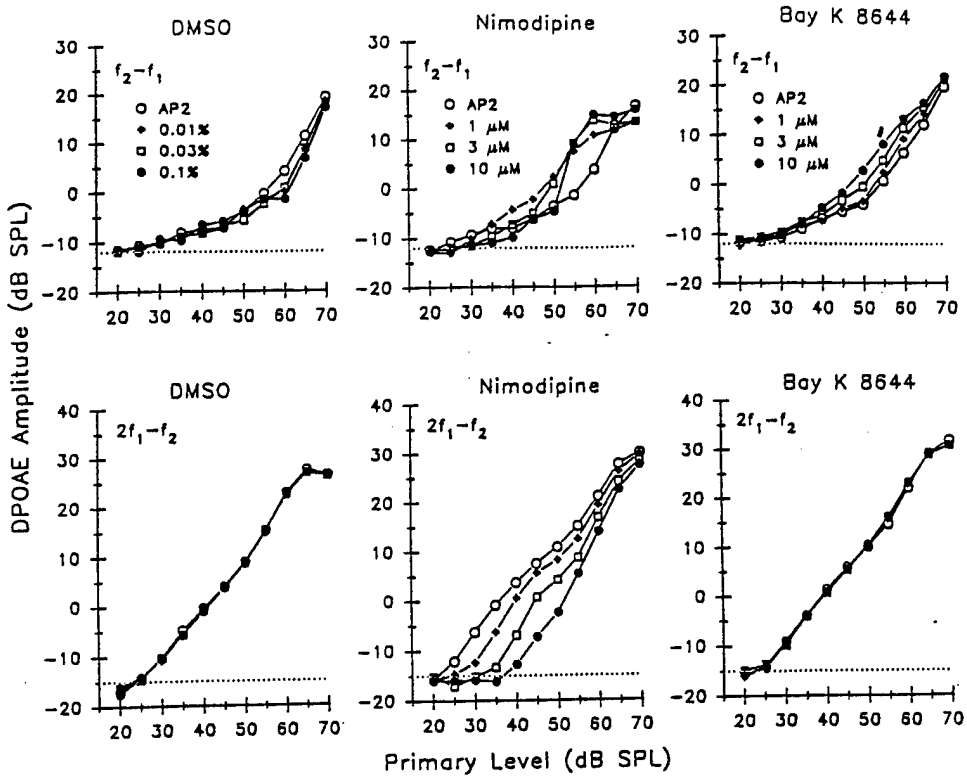

Fig. 5. Effect of perfusion of selected concentrations of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on growth functions for f_2-f_1 and $2f_1$-f_2 DPOAEs. Data are represented as means ($n = 4$ animals per drug treatment; see Figs. 4 and 6 for representative SE and statistics). The dashed line in each panel represents the average value of the noise floor.

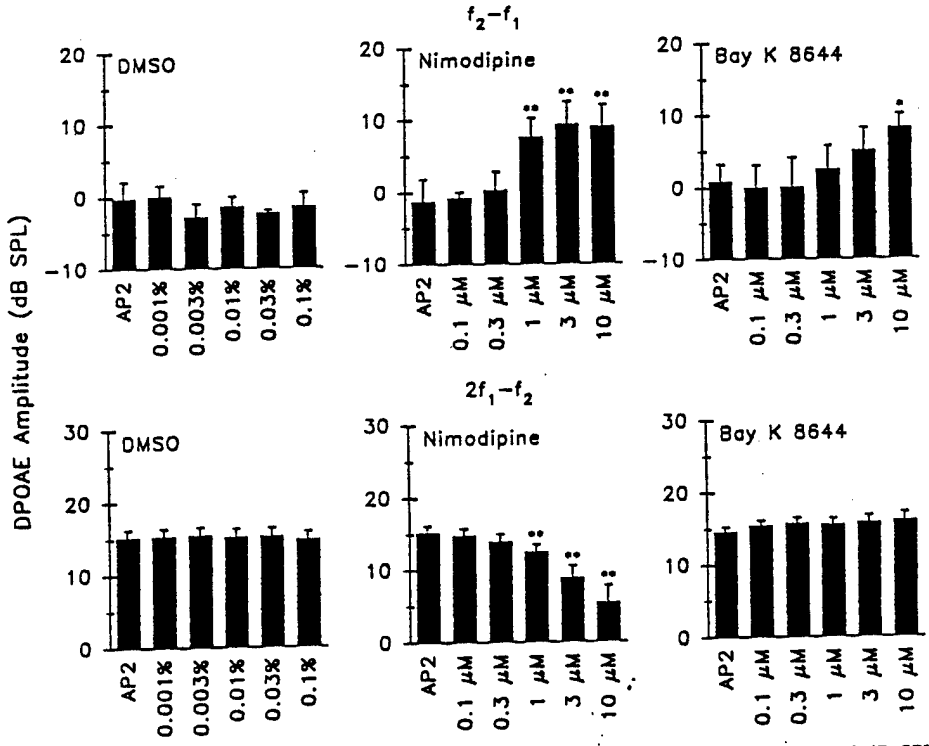

Fig. 6. Effect of perfusion of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO on DPOAE amplitude to 55 dB SPL primaries. The values (means ± SE) are taken from those shown in Fig. 5 and subjected to statistical analysis. Values significantly different from corresponding AP2 are designated: $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 5$ animals per drug treatment).

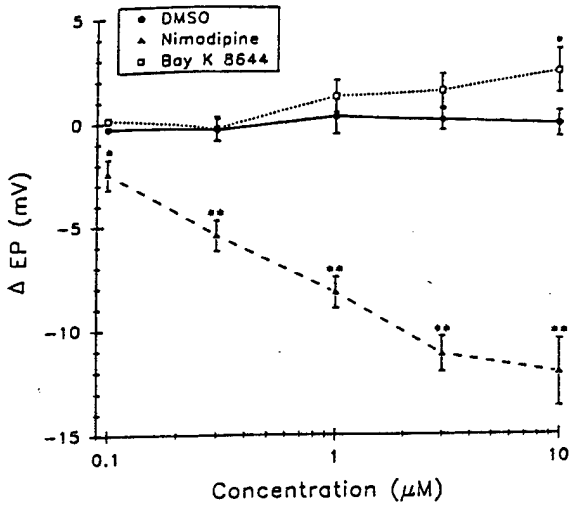

Fig. 7. Dose–response curve of the effects on the EP of DMSO, nimodipine in DMSO and Bay K 8644 in DMSO. Solvent (DMSO) concentrations corresponding to *X*-axis drug concentrations were as follows: 0.1 μM = 0.001%; 0.3 μM = 0.003%; 1.0 μM = 0.01%; 3.0 μM = 0.03%; 10 μM = 0.1%. For each experiment, the last (15 min) EP value obtained during perfusion of the second control perfusion (AP2) was subtracted from the last (15 min) EP value obtained during perfusion of each of the drug concentrations. The means ± SE of each of the values calculated in this manner are shown ($n = 5$ animals per drug treatment). Points significantly different from their respective DMSO-alone controls are designated: $^{*}P < 0.05$; $^{**}P < 0.01$. Average drift in the EP records for each treatment was: DMSO = +3.4 mV; nimodipine = +0.6 mV; Bay K 8644 = +2.2 mV.

ated with its paired or equivalent DMSO concentration at all drug concentrations (0.1–10 μM). An estimated IC_{50} value for nimodipine of 0.42 μM was obtained using a curve-fitting program (Origin®, 4.0). Bay K 8644 induced a small increase in EP that was significantly different only at 10 μM from its paired or equivalent DMSO concentration ($P < 0.05$; Fig. 7).

4. Discussion

4.1. General alterations in DPOAEs by the treatments

The present work demonstrates that perstimulatory changes in the amplitude of the f_2-f_1 DPOAE are altered by perilymph calcium manipulations. Manipulations that lowered available Ca^{2+} (0 Ca^{2+}; 0 Ca^{2+} with BAPTA) or that blocked the entry of Ca^{2+} into the cell through L-type Ca^{2+} channels (nimodipine) were associated with qualitatively similar alterations to the f_2-f_1 DPOAE. Specifically, overall amplitude of the f_2-f_1 DPOAE was reduced, as were the time-varying amplitude alterations during continuous stimulation. For the more potent manipulations (BAPTA; nimodipine), these amplitude changes ultimately reversed direction. Following perfusion of an L-type Ca^{2+} channel agonist (Bay K 8644) overall response amplitude was increased and the slope of the subsequent perstimulatory decline was reduced. Replacement of Ca^{2+} with Mg^{2+} did not reverse the effects, suggesting that these consequences of perilymph Ca^{2+} manipulation are not merely divalent cation effects. Growth functions for the f_2-f_1 DPOAE were altered more substantially than were growth functions for the distortion component at the frequency $2f_1$-f_2. Thus, in contrast to previous suggestions that the f_2-f_1 DPOAE is relatively insensitive to cochlear insult (see Brown, 1993 for review), we found it to be more vulnerable to the perilymph Ca^{2+} manipulations introduced in these experiments.

4.2. Sites and mechanisms underlying response to treatments

The cochlear mechanical response to low-level sound is thought to reflect the contribution of active and physiologically vulnerable motions of the OHCs (Robles et al., 1991; Mammano and Ashmore, 1993). DPOAEs are a reflection of non-linearities in that mechanical response (Robles et al., 1991) and may be the direct result of non-linearities in OHC length changes (Hu et al., 1994) or OHC channel gating mechanisms (Jaramillo et al., 1993). Thus, it seems reasonable to speculate that the alterations in f_2-f_1 amplitude observed during continuous primary stimulation are reflections of local, adaptive processes involving the OHC response to sustained stimulation and the effects observed subsequent to perilymph Ca^{2+} manipulations are related to alterations in these processes. There are several ways (either individually or in combination) that this could occur: (1) through alteration of a Ca^{2+}-dependent motility of the OHCs, (2) through an alteration to the Ca^{2+}-dependent adaptation of the OHC transduction current and (3) through an action of the drugs on the EP, secondarily altering OHC response characteristics.

4.2.1. Alterations to OHC motility

It is well known that isolated OHCs are capable of responding to applied AC electrical stimulations with 'fast' length changes that do not directly require Ca^{2+} or ATP (e.g., Ashmore, 1987). The tonic, DC length changes that can be observed during electrical and mechanical stimulations in vitro, however, appear to be much more vulnerable to insult (Evans et al., 1991) and dependent on metabolic support (Canlon and Brundin, 1991). Parallels between these non-linearities in OHC length changes and other non-linearities of the cochlear response (e.g., summating potential, SP; DPOAE) have been noted (Canlon and Brundin, 1991; Evans et al., 1991; Brown, 1993). In particular, at whatever level they are recorded, the non-linearities appear more sensitive to a variety of cochlear insults including pharmacologic manipulations and acoustic overstimulations (see Evans et al., 1991 for discussion). Of importance here, previous work from this laboratory (Bobbin et al., 1991) demonstrated a greater effect of

perilymph Ca^{2+} manipulations on the SP than on the CM. In those experiments, lowering perilymph Ca^{2+} reduced the magnitude of the SP and increasing perilymph Ca^{2+} augmented the SP. In addition, the L-type Ca^{2+} channel antagonist, nimodipine, reduced the magnitude and reversed the polarity of the SP (Bobbin et al., 1990). These are precisely the same effects observed on the non-linearity of the cochlear response under study in the present experiments — the f_2-f_1 DPOAE. An opposite effect, that of an increase in the magnitude of the f_2-f_1 DPOAE, was observed following perilymph perfusion of the L-type Ca^{2+} channel agonist, Bay K 8644. It is possible that all of these effects are secondary to Ca^{2+}-dependent changes in the non-linear motile response of OHCs and that these effects are mediated through an action at L-type Ca^{2+} channels.

An L-type Ca^{2+} channel has been described electrophysiologically and pharmacologically on OHCs of chick (Fuchs et al., 1990) and guinea pig (Nakagawa et al., 1991, 1992; Chen et al., 1995). These voltage-dependent Ca^{2+} channels are involved in a variety of cellular functions, including activation of contractile machinery in muscle cells and exocytotic secretion from endocrine cells and some neurons (see Kass, 1994 for review). L-type Ca^{2+} channels probably comprise the majority of the voltage-gated Ca^{2+} channels of guinea pig OHCs (Nakagawa et al., 1991; Chen et al., 1995).

It is generally assumed that the electromotile response of in vitro OHCs is driven by changes in membrane potential (e.g., Santos-Sacchi and Dilger, 1988). In vivo, under normal conditions of stimulation, it is likely that the OHC mechanomotile response to stereocilia deflection also will result from, or at least be strongly influenced by, changes in membrane potential (see Kolston, 1995). One difficulty with the hypothesis that L-type Ca^{2+} channels, in particular, are responsible for effects seen on the perstimulatory f_2-f_1 amplitude alterations is the level of depolarization required to activate the channels. Chen et al. (1995) reported that, in guinea pig OHCs studied in vitro, the L-type Ca^{2+} channel opened at roughly -30 mV. Because the normal resting membrane potential of in vivo OHCs is roughly -70 mV and sound-evoked depolarization of in vivo OHCs is usually reported to be less than 10 mV (e.g., Dallos and Cheatham, 1992), it is possible that the threshold of activation of the L-type Ca^{2+} channels would not be reached. However, Evans et al. (1991) note that, at least in vitro, the DC component of the motile response is sensitive to small depolarizations that do not necessarily alter the AC component. It is also possible that the activation curve reported for L-type Ca^{2+} channels in vitro is different in vivo.

It should be noted that nimodipine and Bay K 8644 can exert non-specific effects at ion channels other than L-type Ca^{2+} channels. Lin et al. (1995) recently reported that both of these agents can inhibit a late K^+ current in isolated OHCs maintained in Ca^{2+}-free media (0 Ca^{2+} with EGTA or BAPTA in external and internal solutions). In those studies, however, both nimodipine and Bay K 8644 produced the same effect (inhibition) which is not consistent with the effects of these drugs on the responses monitored in our experiments. Moreover, these effects were obtained at drug concentrations approaching the highest of those tested in the present experiments (IC_{50}s were 6 and 8 μM for nimodipine and Bay K 8644, respectively).

4.2.2. Alterations to Ca^{2+}-dependent adaptation of the OHC transduction current

An additional mechanism that could account for the results obtained is an alteration in processes related to adaptation of the OHC transducer channels. Transducer current adaptation during sustained stimulation is Ca^{2+}-dependent (Eatock et al., 1987; Assad et al., 1991). If the perstimulatory decline in f_2-f_1 DPOAE amplitude is related to these adaptive processes, reducing available Ca^{2+} should reduce the decline. This, along with an actual reversal of the decline, was the observed effect of perfusion with the Ca^{2+} chelator, BAPTA in 0 Ca^{2+} and with the L-channel antagonist, nimodipine.

According to Crawford et al. (1991), transducer current adaptation may be altered either by changes in the endolymph Ca^{2+} concentration or through changes in intracellular Ca^{2+} concentration. With regard to the former, Tanaka and Salt (1994) reported that cochlear function, as reflected in the EP, SP and CAP, was highly sensitive to small disturbances in Ca^{2+} concentration in endolymph. Ikeda et al. (1987) have suggested that, under normal conditions, the Ca^{2+} in endolymph is actively transported from perilymph. Thus, under conditions of reduced perilymph calcium, the concentration of Ca^{2+} at the stereocilia might be altered. Evans and Dallos (1993) have shown that conditions that interfere with mechanotransduction at the level of the stereocilia also interfere with the mechanomotile response of OHCs. Such responses of OHCs in a microchamber to stereocilia displacement disappear rapidly following exposure of the apical portions of the cells (cuticular plate and stereocilia) to an extracellular solution containing no added Ca^{2+} with 20 nM BAPTA. We consider it unlikely, however, that such alterations are responsible for the effects seen in the present experiments. Mechanotransduction can be maintained even with extremely low endolymph Ca^{2+} levels (Crawford et al., 1991) and, in vivo, there are a number of potential tissue pools for Ca^{2+} should such a reduction in endolymph Ca^{2+} occur. Moreover, nimodipine and Bay K 8644 should not affect overall Ca^{2+} levels in the endolymph or in other tissues, as the ion levels are controlled by transport and diffusion. Rather, the drugs likely act on proteins, possibly L-type Ca^{2+} channels.

Adaptation of the transduction current also can be affected at an intracellular site by Ca^{2+} that has entered the cell through a transducer or other membrane channel (possibly a voltage-gated Ca^{2+} channel in the basolateral

membrane; Crawford et al., 1991). These investigators have speculated that during prolonged depolarizations, voltage-sensitive Ca^{2+} channels could be activated. Should the Ca^{2+} that enters via those channels gain access to the transduction channels, adaptation would be facilitated. In this case, the effect on transducer current adaptation would not require an alteration in endolymph Ca^{2+}.

Finally, adaptation could be altered by processes that change the operating point of the stereocilia to mechanical stimulation. Zenner and Ernst (1993) have suggested that, by altering the position of the reticular lamina, DC OHC length changes could influence the operating point of the stereocilia. This, in turn, would modify their sensitivity, possibly altering transduction channel gating and contributing to such processes as adaptation during sustained stimulation and the temporary changes in sensitivity that are associated with overstimulation.

4.2.3. Alteration of the EP

The finding of drug effects on the EP complicates the interpretation of these results. Others have shown that distortion products recorded in the ear canal are sensitive to alterations in the magnitude of the EP (e.g., Mills et al., 1993). This sensitivity is related to the fact that the EP is necessary to power the OHC motile response (Dallos and Evans, 1995). In association with manipulations of the EP, Mills et al. (1993) observed a reduction or a reversal in the magnitude of the f_2-f_1 DPOAE similar to the effects we observed subsequent to perilymph Ca^{2+} manipulations. These investigators report that the magnitude of the $2f_1$-f_2 DPOAE is proportional to the square of the EP. We do not know whether the small changes in EP we observed following perfusion of Bay K 8644 (+2.3 mV) and nimodipine (−12 mV) are sufficient to account for the substantial changes in the responses we monitored, especially since the changes induced by Bay K 8644 are of approximately the same magnitude as the drift in the recordings (see legend, Fig. 7).

In most reports, merely altering perilymph Ca^{2+} levels does not induce changes in EP (Konishi and Kelsey, 1970; Sato, 1989; Bobbin et al., 1991). As noted previously, there are large potential pools for Ca^{2+} in the many fluid compartments of the cochlea, including blood vessels and cerebral spinal fluid entering via the cochlear aqueduct. Given this, it is not surprising that perfusion of low levels of Ca^{2+} through the perilymph compartment would have little impact on the function of the cochlea or on EP magnitude. Even the addition of Ca^{2+} chelators such as EGTA to the perfusate seems to have little effect on the EP (2 nM, Bobbin et al., 1991; 4 nM, Konishi and Kelsey, 1970; 4 and 10 nM, Sato, 1989). Perfusion of the Ca^{2+} chelator, BAPTA, nevertheless, had substantial effects on the continuous stimulation-related changes in f_2-f_1 amplitude recorded in the present experiments.

Nimodipine and Bay K 8644 also altered the perstimulatory amplitude changes in the f_2-f_1 DPOAE and both of these drugs had effects on the EP. Nimodipine and Bay K 8644 exert potent effects on L-type Ca^{2+} channel activity even in the presence of high levels of Ca^{2+} (Chen et al., 1995). Thus, they would not be affected by the large pools of Ca^{2+} in the tissue. Nimodipine significantly reduced the EP at the lowest concentration tested (0.1 μM), and effects increased with increasing drug concentration. At the highest concentration tested (10 μM), the EP was reduced by 12 ± 1.2 mV. This value is in good agreement with the 15 ± 3 mV reduction in EP associated with a single 30 min perfusion of 10 μM nimodipine reported previously (Bobbin et al., 1991). To our knowledge, there are no reports of the existence of L-type Ca^{2+} channels in the marginal cells of the stria vascularis, the structure generating the EP (e.g., Sunose et al., 1994). If these effects on the EP are due to drug actions at L-type Ca^{2+} channels, their location(s) remain to be determined. It is possible that L-type Ca^{2+} channels located at a site remote from the stria affected the EP. For instance, blockade of L-type Ca^{2+} channels in the OHCs by nimodipine may have induced the reduction in EP. Conversely, Bay K 8644 may have increased the EP by 'activating' an L-type Ca^{2+} channel on the OHCs (Chen et al., 1995).

5. Conclusions

Evidence that the amplitude alterations under study in the present experiments are not neurally mediated in general, and not efferent mediated in particular, has been reviewed (Kujawa et al., 1995). Briefly, these alterations in f_2-f_1 DPOAE amplitude were not prevented by the intracochlear application of TTX, which should block all action potential-mediated activity, and they were not prevented by antagonists of MOC efferent activity or by midline section of ipsilaterally responsive MOC neurons. However, efferent activity certainly can alter DPOAEs (e.g., Mountain, 1980; Siegel and Kim, 1982; Kujawa et al., 1993Kujawa et al., 1994●). Of importance here, activation of ipsilaterally responsive MOC neurons by the primaries themselves has recently been reported to result in a rapid (time constant ≈ 100 ms) alteration in the amplitude of the $2f_1$-f_2 DPOAE (Liberman et al., 1996). Such response alterations disappeared immediately upon section of the crossed olivocochlear pathways and are clearly different from the very slow amplitude changes (time constant ≈ minutes) described in the present report.

In summary, previous studies from this laboratory (Bobbin et al., 1990; Bobbin et al., 1991) demonstrated that both lowering perilymph Ca^{2+} levels and perilymph application of the L-type Ca^{2+} channel antagonist, nimodipine, decrease the magnitude, and even reverse the sign of the DC receptor potential known as the SP. Results presented here extend this observation to DPOAEs, in particular the slow amplitude alterations in the f_2-f_1 DPOAE that are associated with sustained primary stimu-

lation. We have speculated previously that such amplitude changes reflect local, Ca^{2+}-dependent, adaptive processes occurring at the level of the OHCs. The present results support that hypothesis and suggest, further, that the effects may be mediated, at least in part, by L-type Ca^{2+} channels. Further study will be required to clarify the site(s) of action of nimodipine and Bay K 8644 in the cochlea.

6. Unlinked lists

Assad et al., 1991, Assad and Corey, 1992, Lin et al. (1995, Kujawa et al., 1994a,b,Santos-Sacchi and Dilger, 1988, Liberman et al., 1995

Acknowledgements

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APPENDIX # 4
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Bobbin and Berlin

Chemical Receptors on Outer Hair Cells and Their Molecular Mechanisms

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INTRODUCTION

We know that normal hearing depends on the proper functioning of active and passive cochlear mechanics and that the outer hair cells (OHCs) play a central role in the active mechanics. Various investigations have shown that the efferent nerves that innervate the OHCs influence cochlear mechanics by way of neurotransmitter chemicals released onto the OHCs. In addition, other cells in the organ of Corti and even the OHCs themselves may also release chemicals that act on the OHCs, that is, the chemicals act in a paracrine or autocrine manner. This chapter reviews the mechanisms of action of a few endogenous chemicals that act on OHCs with emphasis on the unique pharmacology of the efferent neurotransmitter, acetylcholine. The eventual goal of such studies is to understand the action of these molecules at the cellular and subcellular level and so understand the molecular mechanisms of cochlear mechanics.

Active and Passive Mechanics

The function of the cochlea is now thought of in terms of an active and passive mechanism as shown schematically in Figure 2–1. The passive mechanism is utilized during sound exposure over 40–60 dB SPL, where sound energy is powerful enough to move the cochlear partition directly.

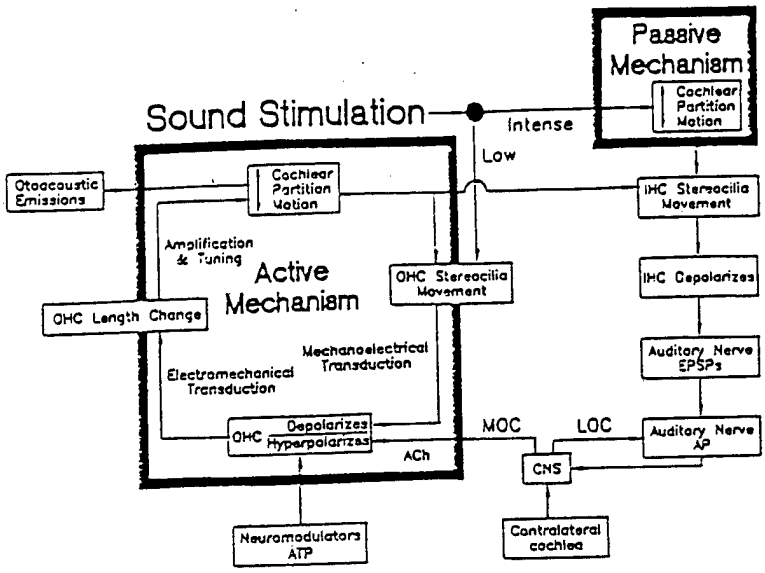


Figure 2–1. Schematic of the action of the passive and active mechanism for cochlear partition mechanics. IHC: inner hair cell; OHC: outer hair cell; LOC: lateral olivocochlear nerve tract; MOC: medial olivocochlear nerve tract; EPSPs: excitatory postsynaptic potentials; AP: action potential; ATP: adenosine triphosphate; ACh: acetylcholine; CNS: central nervous system. (Adapted from Pujol, 1990.)

This results in the movement of inner hair cell (IHC) stereocilia and opening of transduction channels which results in depolarization (less negative or more positive voltage) of the IHCs. Depolarization of the IHCs releases the neurotransmitter (i.e., glutamate) onto the auditory nerve endings which produces action potentials in the auditory nerve fibers. The active mechanism is utilized during low levels of sound exposure (<40 dB SPL) when the sound energy is insufficient to move the cochlear partition directly. Instead, in some unknown fashion, sound at this low level induces movement of the stereocilia on the OHCs. The movement of the OHC stereocilia opens transduction channels and depolarizes the OHCs (i.e., mechanoelectrical transduction). Depolarization of the OHCs changes the length of the OHCs (i.e., electromechanical transduction). This length change then results in amplification by inducing additional movement of the OHC stereocilia and greater depolarization and even greater length change of the OHC. The change in length of the OHC will move the cochlear partition and the greater the OHC length change, the greater the cochlear partition motion. When the movement of the partition is sufficient to induce IHC stereocilia movement, then events as described previously for the passive mechanism will take place, producing action potentials in

the auditory nerve. The amplification of the cochlear partition movement by the active mechanism contributes to the energy being transmitted back out through the ossicles in the form of otoacoustic emissions.

The OHCs are the key elements in the active process, with possibly some role for other cells such as Deiters cells (Dulon, 1994, Dulon, Blanchet, & Laffon, 1994; Dulon, Moataz, & Mollard, 1993; Moataz, Saito, & Dulon, 1992). Therefore, the OHC is a good anatomical point for the ear to control its response to sound. This is accomplished through several chemicals that act on the OHCs to alter their ability to change length in response to sound. As the motor of the OHCs is voltage controlled (Santos-Sacchi & Dilger, 1988), the degree of polarization of the OHCs determines the length of the OHCs: the more positive and farther away from its resting potential (@ –60 mV) the greater the shortening. Some chemicals depolarize the OHCs (i.e., membrane potential more positive) and this allows the OHC to shorten to a greater degree in response to sound. Others hyperpolarize the OHCs (i.e., membrane potential more negative) and this makes the OHC shorten less to sound. Other chemicals that affect intracellular chemical messengers are called second messengers (e.g., Ca^{2+}). Second messengers increase or decrease the length change in response to sound through a molecular mechanism inside the cell. The most studied chemicals are those released onto the OHCs from the efferent nerve fibers.

Efferent Innervation

Others have described the innervation of the cochlea by the olivocochlear efferent nerve fibers (e.g., Warr & Guinan, 1979). As shown in Figure 2–2, the medial portion of the olivocochlear efferents (MOC) synapse with the

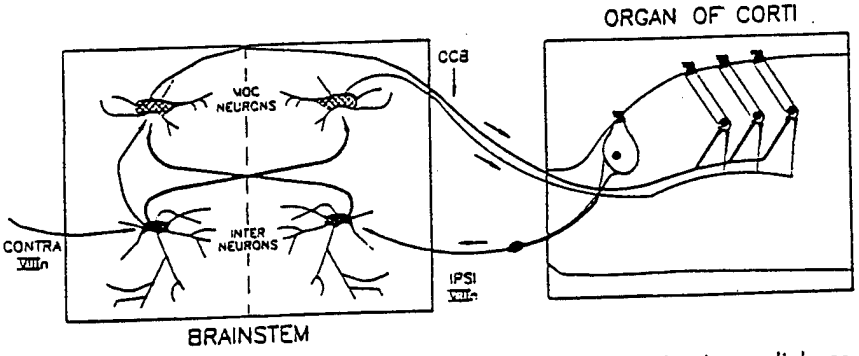


Figure 2–2. Innervation of the organ of Corti and the hair cells by the medial portion (MOC) of the olivocochlear nerve bundle (OCB) which originates in the brain stem. (Adapted from Liberman and T. Kawase, 1992.)

OHCs. Both contralateral and ipsilateral sound activate these MOC efferents and affect the activity of the OHCs by means of released chemical messengers. Thus, MOC nerve fibers play an important role in adjusting the active process. There are thought to be several chemicals involved in the mechanism of action of these efferent nerve fibers (see review by Eybalin, 1993). Here we will discuss only a few of the chemicals, with emphasis on acetylcholine (ACh).

NEUROTRANSMITTERS

Acetylcholine

Antagonists—in Vivo

There is no doubt that acetylcholine (ACh) functions as the primary neurotransmitter that the MOC neurons release onto the OHCs. This evidence comes from the pioneering studies by Guth, Norris, and others (see reviews by Bledsoe, Bobbin, & Puel, 1988; Daigneault, 1981; Eybalin, 1993; Guth, Norris, & Bobbin, 1976). ACh is a neurotransmitter at many locations, for example, the heart, neuromuscular junction. However, it appears that the receptor protein on the OHCs, to which ACh couples, is unique.

The uniqueness of the pharmacology of this ACh receptor protein was suggested in the earliest studies utilizing electrical stimulation of the MOC fibers in the brain stem and applying antagonists of various receptors intracochlearly to the perilymph compartment. Desmedt and Monaco (1960) were the first to demonstrate that strychnine applied to the round window (i.e., into perilymph) blocked the electrically induced action of these efferents (Figure 2–3). They suggested that the receptor protein was similar to the glycine receptor, since at that time strychnine was thought to be a specific blocker of neurotransmitter glycine. However, Churchill, Schuknecht, and Doran (1956) and Schuknecht, Churchill, and Doran (1959) had demonstrated that the efferent fibers stained for cholinesterase, suggesting that the efferent transmitter was ACh. Fex (1968) then demonstrated that curare, an antagonist of the ACh receptor at the neuromuscular junction, applied to the perilymph, blocked the efferents. This was some of the first evidence presented to show that the receptor protein on the OHCs was similar to the ACh receptor protein at the neuromuscular junction (Figure 2–4; because nicotine activates the ACh receptor at the neuromuscular junction, the receptor is called a nicotinic receptor—abbreviated Nm).

Table 2–1 is a partial summary of the pharmacology of this synapse obtained utilizing intracochlear perilymph application of the drugs and

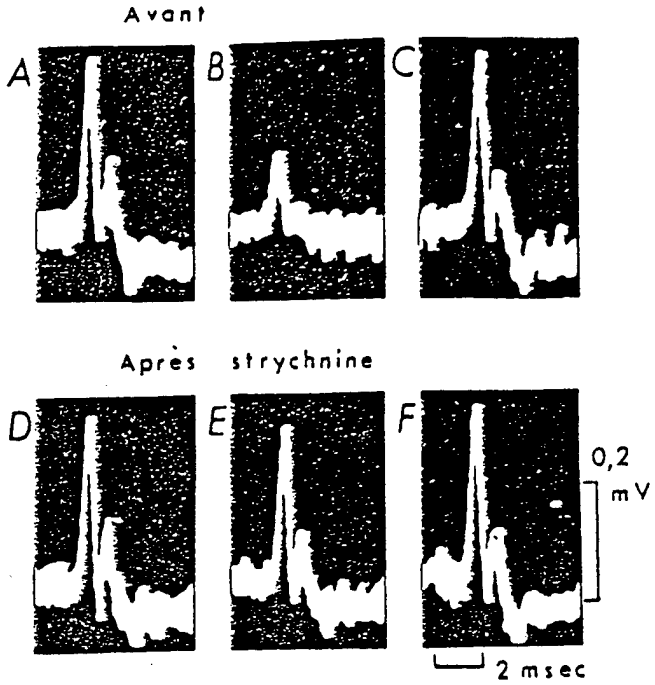


Figure 2–3. Oscilloscope traces showing the responses of the acoustic nerve (round window) stimulated by a click of 20 dB above threshold. Four successive traces are superimposed on each graph in order to show the stability of the response. In B and E, a train of 30 shocks (30 microseconds, 6 volts) at 300/sec was applied to the bundle of Rasmussen at the level of its decussation 20 msec before the tested click. The olivocochlear inhibition of the click response appears clearly when you compare the traces B and E to the responses of a single click (not preceded by the train) obtained before (A and D) and after (C and F) the stimulation of the bundle of Rasmussen. The administration of strychnine considerably reduced the olivocochlear centrifugal inhibition, but does not affect the response to the single click. (From Desmedt and Monaco, 1960, with permission.) (Translated by author)

electrical stimulation of the MOC efferents in the brain stem (from Bobbin & Konishi, 1971a, 1971b, 1974; Fex & Adams, 1978; & Galley, Klinke, Oertel, Pause, & Storch, 1973). For instance, Konishi and I found that the effects of the efferents could be blocked by atropine, a muscarinic receptor antagonist (the receptor found at the autonomic innervation of glands and smooth muscle and called a muscarinic type of receptor as it is activated by the drug muscarine, abbreviated M). Atropine exhibited less potency than either strychnine or curare. At the time, this unusual block by atropine was tempered by the fact that the quaternary atropine was

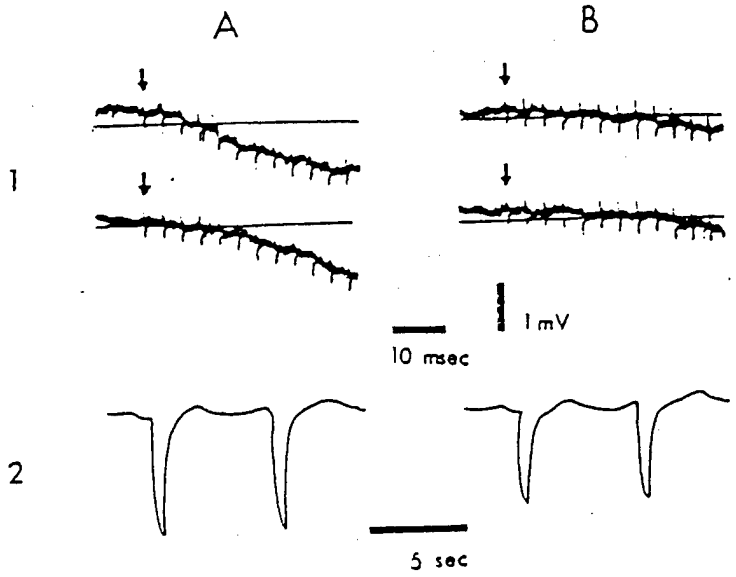


Figure 2–4. Records illustrating the action of d-tubocurarine on centrifugally evoked intracochlear potentials. Row 1 shows the initial time-course of potentials that were evoked by repetitive electrical stimulation of crossed olivo-cochlear fibres. Row 2 shows the full time-course of the potentials of Row 1. All the records were taken with the microelectrode in one position in the scala media. The start of stimulation is indicated by an arrow in Row 1. A. Control, artificial perilymph in the scala tympani. B. 12.5 minutes later than A. Artificial perilymph containing 1.0 μmole (0.7×10^{-6} g/ml) d-tubocurarine had, 5–8.5 minutes later than A, partly replaced the solution without d-tubocurarine. Note that after the application of d-tubocurarine, the time of rise and the latency of the potentials were prolonged and the amplitude decreased.

Note that voltage calibration is common to all the records, while time calibration is different for the two rows. The ink-writer that produced the records of Row 2 had a rise time constant of 0.15 seconds; d.c. recordings were used for all records. Negativity is downward. (From Fex, 1968, with permission.)

more potent than the tertiary atropine, a finding in harmony with a nicotinic type of receptor. In addition, we found decamethonium more potent than hexamethonium, suggesting that the receptor was like the acetylcholine receptor at the neuromuscular junction (nicotinic receptor, Nm) and less like the acetylcholine receptor at autonomic ganglia (nicotinic, Nn). Others reported that alpha-bungarotoxin, an antagonist at the Nm

TABLE 2–1. A summary listing of antagonists of the effects of electrical stimulation of the MOC on cochlear potentials. Also given is a list of the receptor types and the locations where these antagonists are most selective.

Receptor type	Location	Antagonist of MOC
Nicotinic (Nm)	skeletal muscle	curare, α-BTX, quaternary atropine, decamethonium
Nicotinic (Nn)	autonomic ganglia	hexamethonium
Muscarinic (M)	smooth muscles, glands	atropine
Glycinergic (gly)	central nervous system	strychnine

receptor, also blocked the efferents (Fex & Adams, 1978). Overall, it appeared that the receptor protein on the OHCs was similar to the Nm receptor, yet it was different because it was blocked by so many drugs that acted at other receptors. But because the experimental preparations were so complex, one was not sure where the drugs were acting. For instance, the action of strychnine was attributed to the drug blocking the release of ACh and not due to blocking the receptor (Fex, 1968, p. 183).

An additional problem with studying this efferent/OHC synapse using electrical stimulation of the MOC nerves was the difficult surgery and the instability of the preparation during the experiment. The discovery by Puel and Rebillard (1990) of a method of activating the efferents by sound to the contralateral ear yielded a technique that made the study of the pharmacology much easier. They monitored the effect of the efferents on the distortion product emissions (DPOAEs) which reflect the activity of the OHCs, and demonstrated that DPOAEs were suppressed by contralateral sound (Figure 2–5).

Kujawa, Glattke, Fallon, and Bobbin (1993) duplicated the Puel and Rebillard study and found that the response to contralateral noise was blocked by intracochlearly applied strychnine, curare, and atropine, with atropine being the least potent (Figure 2–6). In addition, the preparation was sufficiently stable so that Kujawa, Glattke, Fallon, & Bobbin (1994a) could generate cumulative dose response curves for the various types of pharmacological agents (Figure 2–7). Utilizing this technique, Kujawa et al. (1994a) demonstrated that the ACh receptor on the OHCs did indeed

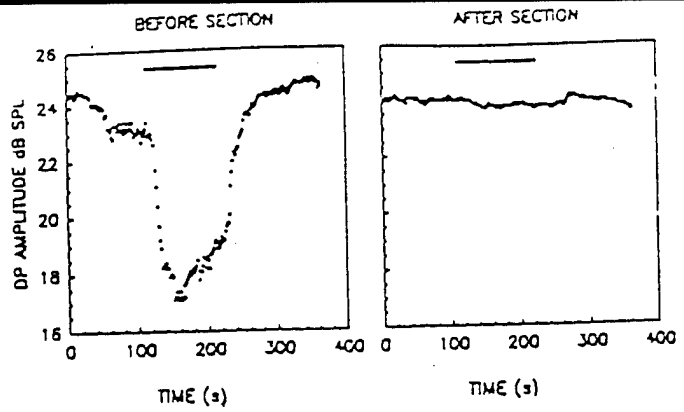


Figure 2–5. Effect of a midline sagittal section of the brainstem on 2F1–F2 DPs recorded at 5 kHz. The left panel represents the 2F1–F2 DPs reduction induced by a 100 dB SPL contralateral white noise. The right panel shows that after a complete midline sagittal section of the brainstem, the suppressive effect of the contralateral white noise is no longer active. The horizontal bars represent the time during which the contralateral white noise was presented. (From Puel and Rebillard, 1990, with permission.)

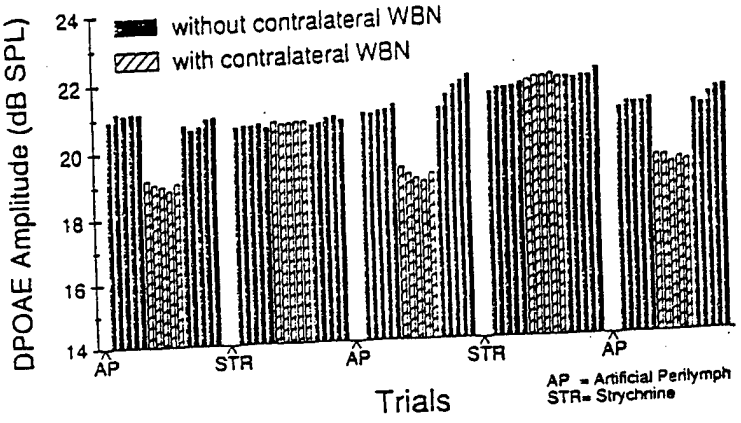


Figure 2–6. Effect of intracochlear strychnine on contralateral suppression of DPOAEs in one representative animal. DPOAE amplitudes are shown after the second control perfusion (artificial perilymph; AP) and after perfusions of 10 μM strychnine (STR) which were followed by artificial perilymph (AP) perfusions that washed out the drug. Each perfusion was 10 minutes in duration; post-perfusion measures are separated by an approximately 25 minute interval during which time the perfusion was completed, post-perfusion measures were taken, and the perfusion pipette was prepared for the next perfusion. Each "trial" represents a 50 spectra average and required 20 seconds to complete. Each set of post-perfusion measures thus required a total of approximately 300 seconds. Graph represents a total time of approximately 2.5 hours. (From Kujawa et al., 1993, with permission.)

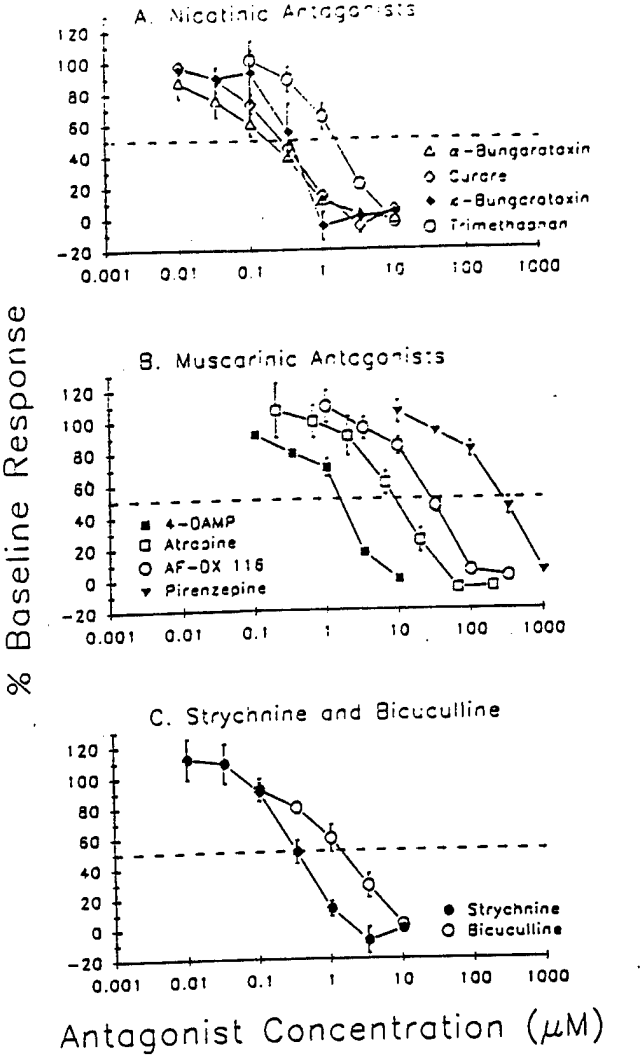


Figure 2–7. Inhibition curves (means ± S.E.) for antagonist blockade of baseline contralateral suppression. Curves associated with nicotinic antagonists are displayed in panel A, those associated with the muscarinic antagonists in panel B and the curves associated with the nontraditional cholinergic antagonists strychnine and bicuculline are shown in panel C. Magnitude of contralateral suppression following the second control perfusion established the baseline response for each animal. Magnitude of suppression as recorded following each concentration of experimental drug was then expressed as a percentage of this baseline response. Horizontal line in each panel designates the point of 50% blockade of baseline suppression (IC_{50}). For all drugs but κ-bungarotoxin, each set of means is based on $N = 5$ animals; for κ-bungarotoxin, $N = 2$ animals. (From Kujawa et al., 1994a, with permission.)

appear to have an unusual pharmacology: it was readily blocked by strychnine and by ACh antagonists. The drugs active at the neuromuscular junction (nicotinic, Nm, e.g., curare) were more potent than those active at autonomic ganglia (nicotinic, Nn, e.g., trimethaphan) or at smooth muscle (muscarinic, M, e.g., atropine). Kujawa et al. added another unusual property of the receptor: a block by the GABA receptor antagonist, bicuculline. Again because of the complexity of the preparation, each drug's exact site of action remains obscure, but the unusual pharmacology obtained with electrical stimulation was confirmed utilizing natural stimulation of the MOC nerve fibers.

Antagonists—in Vitro

Housley and Ashmore (1991) were the first to demonstrate that the ACh receptor protein's spectrum of pharmacology to ACh antagonists at the level of the isolated OHC was similar to that obtained using the whole animal (Figure 2–8; potency in blocking applied ACH: curare > strychnine > atropine > pirenzepine). Fuchs and Murrow (1992b) demonstrated a similar spectrum of pharmacological activity in the chick hair cells (Figure 2–9; potency: strychnine > timethaphan > curare > atropine). Kakeha-

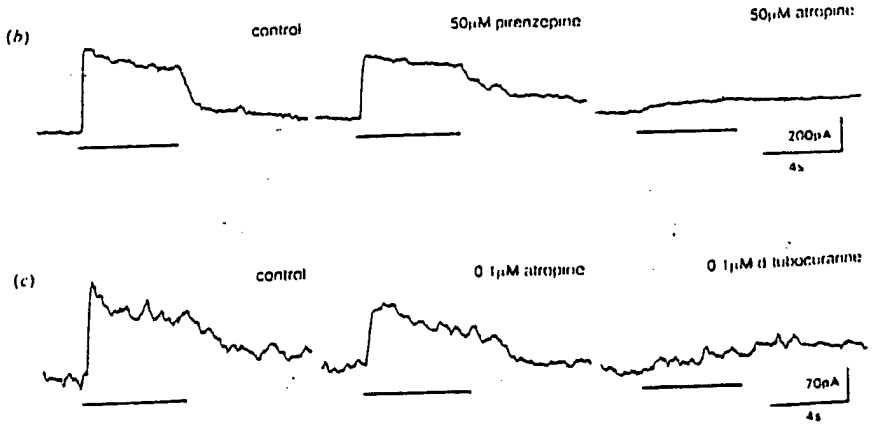


Figure 2–8. Localization and synaptic properties of the ACh response. (a) Not shown. (b) Atropine was more effective than pirenzipine as an antagonist when included at equimolar concentrations with 50 μM ACh. (c) The nicotinic antagonist d-tubocurarine was more effective than atropine when both were applied at 100 nM along with 50 μM ACh; 5 s pressure pulse monitored below. Holding potential, –50 mV in (a) -60 mV in (b, c). (From Housley and Ashmore, 1991, with permission.)

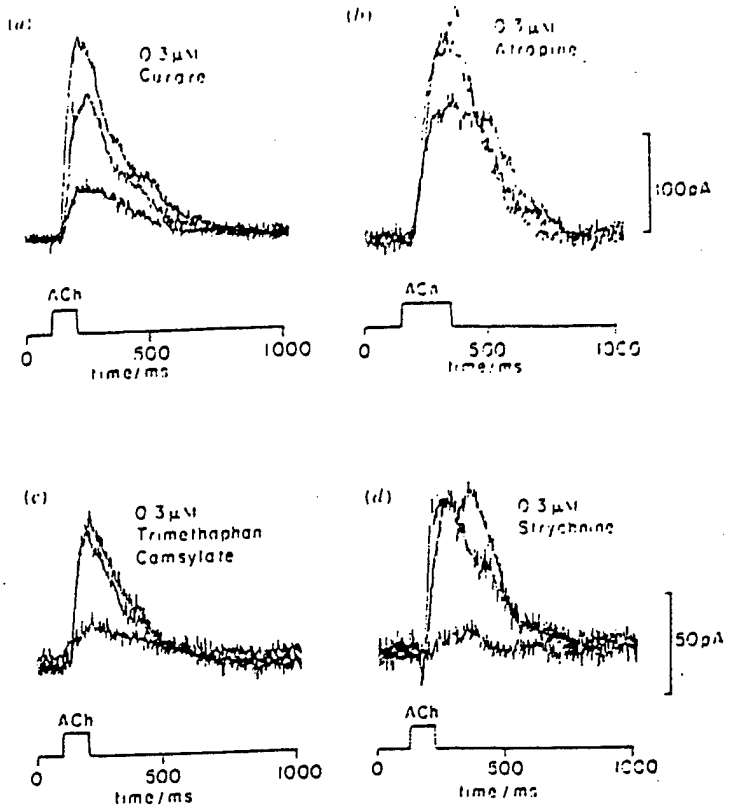


Figure 2–9. Antagonists of the hair cell ACh response. (a) Curare (0.3 μM) blocked 62% of the ACh (100 μM) response. Membrane potential –44 mV. (b) Atropine (0.3 μM) blocked 37% of the ACh response. Membrane potential –24 mV. (c) Trimethaphan camsylate (0.3 μM) blocked 77% of the ACh response. Membrane potential –54 mV. (d) Strychnine (0.3 μM) blocked 79% of the response. Membrane potential –54 mV. Application of ACh indicated by the lower bar. Pre- and post-controls shown in each panel. All antagonists were shown to be completely reversible in these or other cells. (From Fuchs and Murrow, 1992b, with permission.)

ta, Nakagawa, Takasaka, and Akaike (1993) tested muscarinic antagonists and found the following order of potency against applied ACh: atropine = 4-DAMP > AFDX 116 > pirenzepine. Erostegui, Norris, and Bobbin (1994) compared many of the drugs in the same preparation, extending the previous findings and showing that bicuculline blocks the effect of applied ACh (Figure 2–10; Table 2–2). Overall, the data of Erostegui et al. indicate the following order of potency of antagonists against applied ACh: strychnine, a glycine receptor antagonist > curare, a

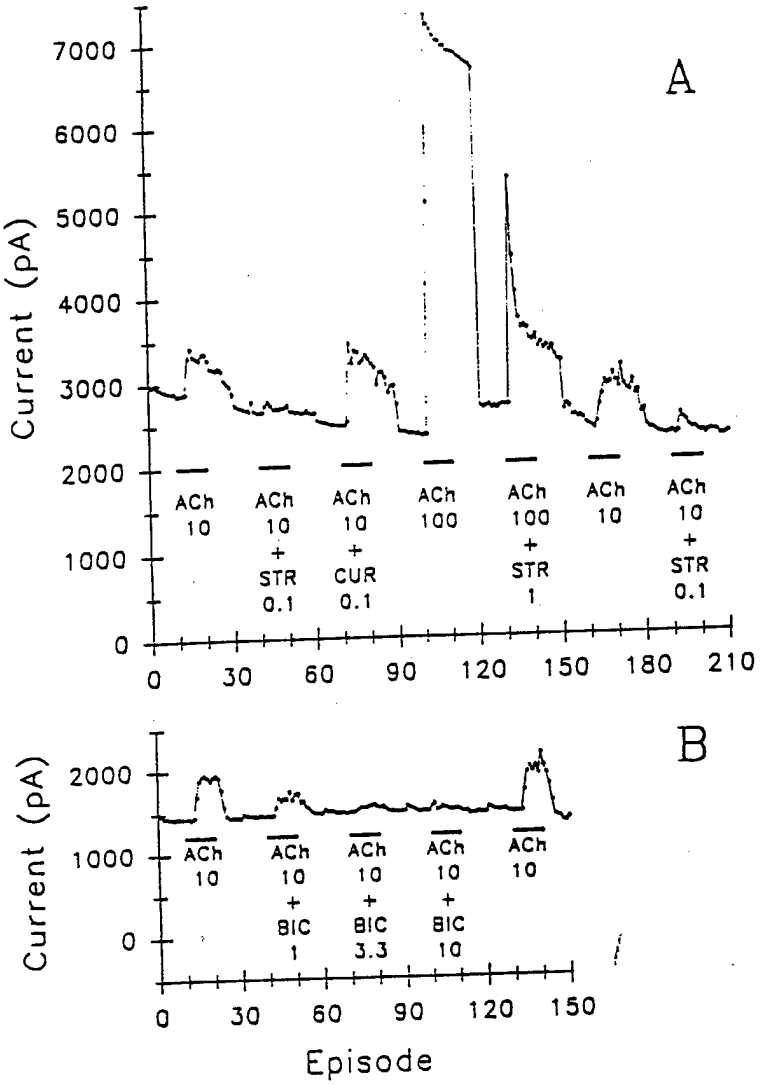


Figure 2–10. Effect of the nontraditional cholinergic antagonists, strychnine (STR) and bicuculline (BIC) on the response to ACh. A: strychnine (0.1 μM) blocked ACh. The large response (4.7 nA) to ACh (100 μM) is suppressed in great extent by strychnine at 1 μM. Bicuculline (3.3 and 10 μM) blocked ACh. Numbers near the drug abbreviations are drug concentrations in μM. Shown are data obtained as voltage clamp current responses at zero mV utilizing the same step protocol as illustrated and described in Figure 2–2. See Figure 2–2 for further description. (From Erostegui et al., 1994, with permission.)

TABLE 2–2. Summary of effects of ACh antagonists on the ACh evoked current response in isolated OHCs.

	Concentration (in millimolar):								
ACh Antagonist	0.1	0.3	1	3.3	10	33	100	330	1000
Curare	0%	–	87%	100%					
Trimethaphan Camsylate	0%	–	40%	100%	100%				
α-bungarotoxin	0%	0%	50%	100%					
Atropine	–	–	0%	45%	100%	100%			
4-DAMP	–	–	0%	25%	66%	100%			
AFDX	–	–	–	0%	44%	100%			
Pirenzepine	–	–	–	–	–	–	50%	100%	100%
Strychnine	75%	–	100%						
Bicuculline	–	–	75%	90%	100%				

Source: Erostegui et al. (1994).

Note: Shown are the concentrations of antagonist (millimolar) tested against the current induced by 10 μM ACh in single isolated OHCs. Percentage indicates the number of cells in which the induced current was blocked out of the number of cells tested with the concentration of antagonist.

Nm receptor antagonist > bicuculline, a GABA receptor antagonist > atropine, a M receptor antagonist. Surprisingly, the pharmacological data obtained in vivo matched fairly closely with the data obtained in vitro. Because these studies used single cells and studied the blockade of applied ACh, the drugs were all acting at the OHC membrane, probably at the ACh receptor protein. However, even single cell experiments are complex; some of the drugs that blocked the action of applied ACh may have been acting at ion channels or other sites on the OHC and not directly on the OHC receptor protein. More studies will have to be done to prove the site of action.

Agonists–in Vivo

The use of antagonists characterizes a receptor protein to a certain extent. On the other hand, agonists can give more information, as these are the chemicals that activate the receptor protein to initiate subsequent cellular events. For instance, when ACh was combined with eserine (which blocked cholinesterase from degrading the ACh molecule) and placed into the cochlear fluids, it not only mimicked the effects of efferent stimulation, but in the face of the continuous application of ACh the response declined or desensitized while concurrently the efferents became ineffec-

tive (Bobbin & Konishi, 1971a, 1971b; Figure 2–11). Kujawa, Glattke, Fallon, and Bobbin (1992) replicated this earlier work by utilizing DPOAEs and again demonstrated desensitization. These studies demonstrated that ACh could act at this synapse in a manner similar to activation of the MOC efferent fibers. In addition, they demonstrated desensitization of the response and emphasized the activity of cholinesterase in destroying applied acetylcholine. More importantly for this discussion, Konishi and I demonstrated that neither nicotine, a powerful agonist at the nicotinic receptors, nor arecoline, an agonist at muscarinic receptors, had much of an effect (Bobbin & Konishi, 1971a, 1974). Galley et al. (1973) described

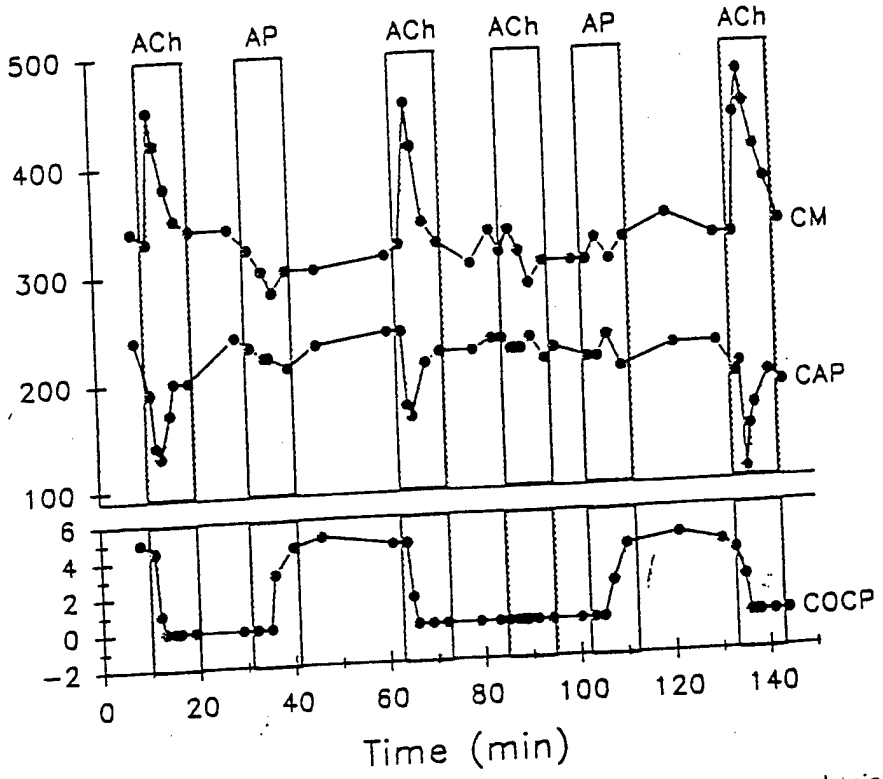


Figure 2–11. Effect of perfusion of the scala tympani on the cochlear microphonic (CM) (600 Hz, 64 dB SPL) and auditory nerve potential (AP) (6 kHz, 81 dB SPL) recorded without COCB stimulation, and the slow potential change (COCP) elicited by COCB stimulation. The scala tympani was perfused with artificial perilymph (A) and with acetylcholine chloride (250 μM) together with eserine sulphate (10 μM; B). (From Bobbin and Konishi, 1971b, with permission.)

the low potency of the muscarinic agonist, muscarone. Thus, even though the data with antagonists demonstrated the receptor was nicotinic (i.e., Nn), the data with agonists suggested the receptor was not nicotinic or muscarinic as the agonists had no effect.

Agonists–in Vitro

Recently, Kakehata et al. (1993) confirmed the in vivo data utilizing individual isolated OHCs. They demonstrated very little or no activity with nicotine, muscarine, McN-A-343, oxotremorine, and oxotremorine-M (Figure 2–12). They found that the most potent agonists were acetylcholine and carbachol. Erostegui et al. (1994) found that not only were nicotine and muscarine ineffective, but so was cytisine (Figure 2–13). On the other hand, Erostegui et al. found that DMPP and suberyldicholine, additional nicotinic agonists, were active.

Table 2–3 summarizes the unique pharmacology of this unusual receptor. The ACh receptor protein appears to have an unusual spectrum of activity in response to the various antagonists; the antagonists listed in Table 2–1 all block the ACh receptor on OHCs even though they are specific for the other receptors listed (e.g., pirenzepine vs the M1 receptor). In addition, the receptor fails to be activated by either the M receptor agonist, muscarine, or the Nn and Nm receptor agonist, nicotine. Cytisine, an additional nicotinic receptor agonist also failed to activate the receptor. On the other hand, it was activated by DMPP and suberyldicholine. To the best of our knowledge, no receptor to date has been described with this spectrum of pharmacological activity (e.g., Seguela, Wadiche, Dineley-Miller, Dani, & Patrick, 1993), although recent results with an alpha 9 receptor subunit come very close (Elgoyhen, Johnson, Boulter, Vetter, & Heinemann, 1994). So it appears that the receptor protein on the OHCs may be unique and not described to date.

In previous publications, I argued that the receptor was nicotinic, but this was based on the pharmacology to the antagonists. On the other hand, it is difficult to name it nicotinic when nicotine has no effect. In general, receptors are named after the drug most active at the receptor, for example, nicotinic or muscarinic. So it seems then that the ACh receptor on the OHC should not be called nicotinic or muscarinic. As suberyldicholine is one of the most potent agonists, the receptor should be called a suberyldicholinic receptor, or "subdic" for short (Bobbin, 1994).

Receptors have been grouped into families based on their molecular configuration. For instance, at present ACh receptors are classified as belonging either to the nicotinic family or the muscarinic family. At present we do not know to which family the ACh receptor on the OHC

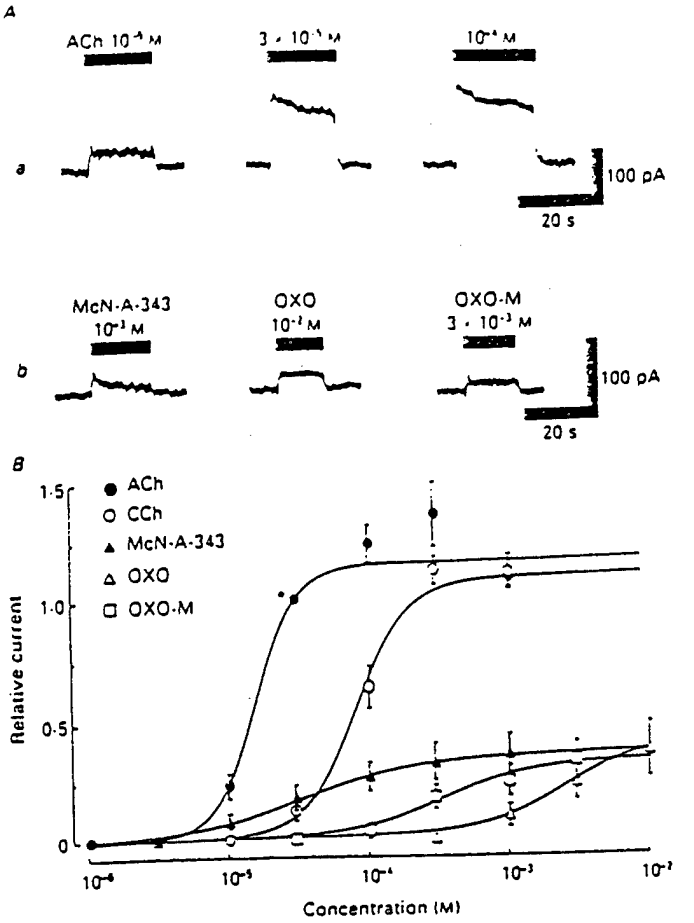


Figure 2–12. I_{ACh} in dissociated outer hair cells (OHCs). A a,I_{ACh} at various concentrations under voltage-clamp conditions using the perforated patch-clamp technique. The holding potential (V_H) was –40 mV. Horizontal bars above each response indicate a period of continuous ACh application. The current was recorded from a 40 μm OHC of the second turn. The resting membrane potential (VR) and the input impedance were –60 mV and 33 MΩ, respectively. The amplitude of the current injected to hold the membrane potential of –40 mV was 600 pA. A b, representative current induced by McN-A-343, oxotremorine (OXO), and oxotremorine-M (OXO-M). The 50 μm cell was obtained from the second turn. The V_R and the input impedance were –62 mV and 67 MΩ, respectively. The amplitude of the current injected to hold the membrane potential of –40 mV was 320 pA. B, concentration-response relationships for ACh, carbamylcholine (Cch), and various muscarinic agonists. Amplitudes of currents induced by each drug at various concentrations were normalized to the current induced by 3×10^{-5} M ACh (*). Each point is the mean ± S.E.M. of four to seven cells. (From Kakehata et al., 1993, with permission.)

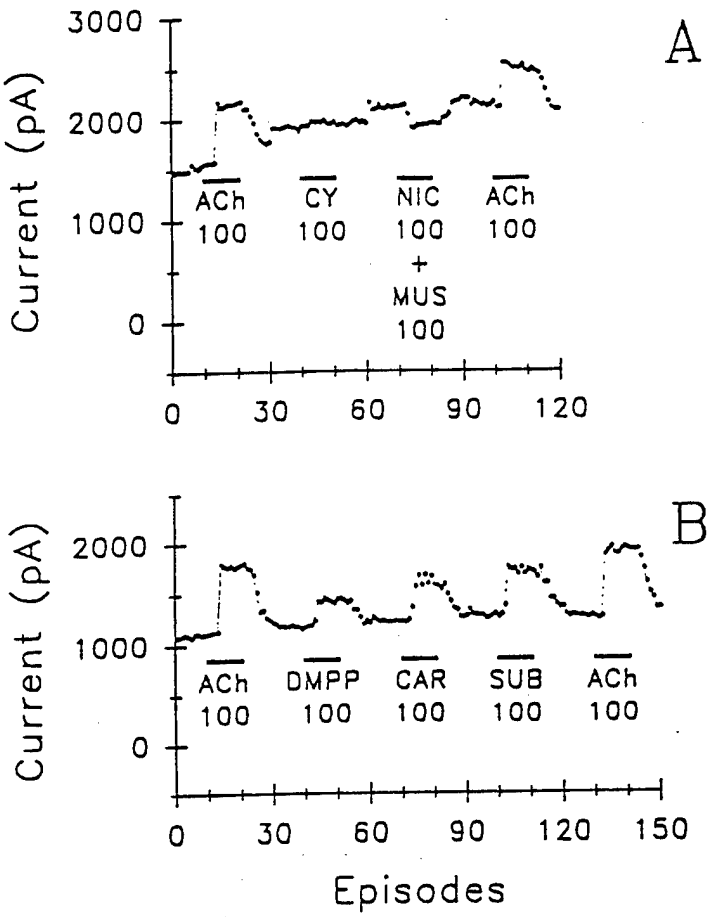


Figure 2–13. Effect of the ACh receptor agonists compared to ACh in two cells; frame A, cytisine (CY) and nicotine with muscarine (NIC + MUS); frame B: DMPP, carbachol (CAR) and suberyldicholine (SUB). In frame A between records (every 30 episodes) an apparent recovery of some current strength occurred as in Figure 2–8. Note the mixture of nicotine with muscarine suppressed the current. Numbers near the drug abbreviation are drug concentrations in μM. Shown are data obtained as voltage clamp current responses at zero mV utilizing the same step protocol as illustrated and described in Figure 2–2. See Figure 2–2 for further description. (From Erostegui et al., 1994, with permission.)

TABLE 2–3. Summary of the various antagonists of ACh at the OHC and a list of the other receptors where these antagonists are thought to be most selective.

Receptor Type[a]	Agonist	Location	ACh Antagonist[b]
suberyldicholine or subdic (S)	Ach, suberyl-dicholine	OHCs	strychnine
nicotonic (Nm)	ACh, nicotine	skeletal muscle	curare, α-BTX
nicotinic (Nn)	ACh, nicotine	autonomic ganglia	trimethaphan
muscarinic (M)	ACh, muscarine	smooth muscle glands	atropine
muscarinic (M_1)	ACh, muscarine	"	pirenzepine
muscarinic (M_2)	ACh, muscarine	"	AF-DX 116
muscarinic (M_3)	ACh, muscarine	"	4-DAMP
glycinergic (gly)	glycine	central nervous system	strychnine
GABAergic (GABA)	GABA	"	bicuculline

[a]Suberyldicholine is the name proposed for the ACh receptor type on the OHCs.
[b]All of the antagonists listed block the effects of ACh at the OHC. Only strychnine is listed at the OHC because it is the most potent antagonist against ACh.

belongs. It is unlikely that ACh belongs to a new family, even though new families of receptors are being discovered (e.g., ATP family; Valera et al., 1994). However, until the molecular composition of the subdic receptor is determined, the "family" will remain unknown.

Adenosine triphosphate (ATP)

ATP was first suggested to be a candidate for a neurotransmitter or neuromodulator in the cochlea based on its relative potency in reducing the compound action potential of the auditory nerve (Bobbin & Thompson, 1978). Subsequent studies have demonstrated that ATP increases intracellular Ca^{2+} levels in inner hair cells (Dulon, Mollard, & Aran 1991) and Deiters cells (Dulon et al., 1993) and has powerful effects on the membrane potential of the OHCs (Ashmore & Ohmori, 1990; Housley, Greenwood, & Ashmore, 1992; Kakehata et al., 1993; Kujawa, Erostegui, Fallon,

Crist, & Bobbin, 1994b; Nakagawa, Akaike, Kimitsuki, Komune, & Arima, 1990). ATP activates a receptor on OHCs that induces a large inward current and so depolarizes the OHC.

ATP is metabolized very rapidly to adenosine, a compound with little activity in the cochlea. However, chemists have made a few ATP analogues that are not as rapidly metabolized, such as ATP-γ-S. Recently, Kujawa et al. (1994b) demonstrated that ATP-γ-S was one of the most potent compounds studied when instilled into the perilymph: ATP-γ-S abolished the CAP and the DPOAEs, and decreased the low intensity sound-evoked SP while it increased the high intensity sound-evoked SP (Figure 2–14). In addition, Kujawa, Fallon, and Bobbin (1994c) demonstrated powerful effects of ATP antagonists on cochlear potentials suggesting a role for endogenously released ATP in normal physiology.

The role of ATP and its receptor proteins in the cochlea is unknown. It may have both a paracrine and an autocrine role. Some suggest ATP is released from the efferent nerve fibers and as such is utilized as a depolarizing agent to counter the hyperpolarizing effects of ACh. Others suggest it may be acting on the scala media side of the OHC (near the stereocilia) to regulate the polarization of the OHC (Housley et al., 1992). In addition, ATP may have a role in programmed cell death (Valera, Hussy, Evans, Adami, North, Surprenant, & Buell, 1994). In summary, ATP has powerful effects in the cochlea, but the role of these effects in the physiology of the cochlea remains to be determined.

Other chemicals

GABA appears to be a transmitter at a small number of efferent nerve fibers that synapse on OHCs (see review by Eybalin, 1993). GABA may allow ions such as chloride to enter the cell and so hyperpolarizes the OHC (Gitter & Zenner, 1992).

Additional chemicals have been suggested to have a possible role at the OHCs. Glutamate, which is the transmitter between IHCs and the afferent nerves, and probably the transmitter between the OHCs and their afferent nerves (Bobbin, 1991) when applied acutely has no effect on the currents recorded from the OHCs in the whole cell voltage clamp configuration (Chen & Bobbin, 1994). However, glutamate may have a role on second messenger chemicals inside the OHCs that we cannot detect by measuring acute changes in cell current. Calcitonin-gene-related-peptide (CGRP) is another chemical found in the efferents innervating the OHCs for which a role has yet to be determined (Eybalin, 1993).

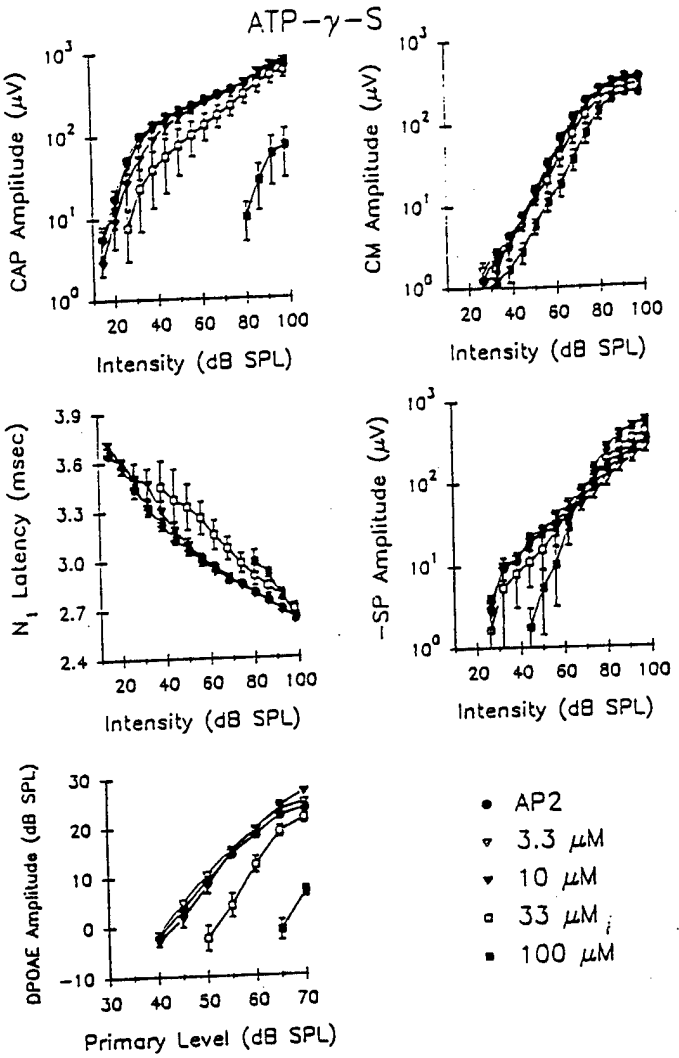
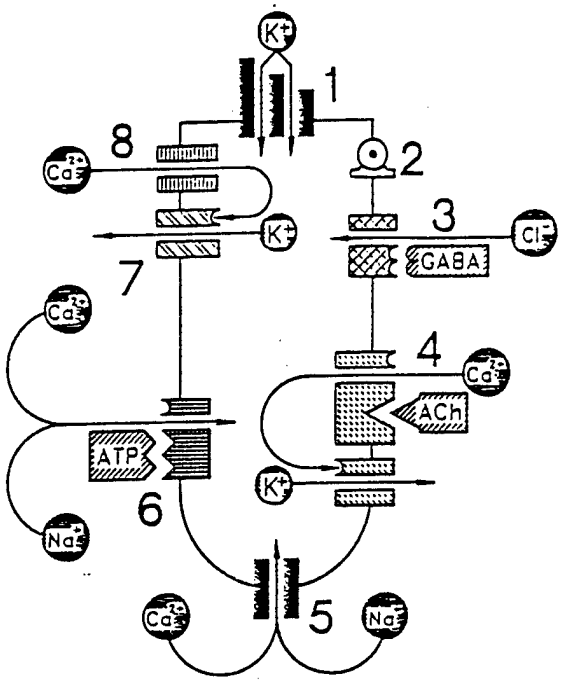


Figure 2–14. Effect of ATP-γ-S on CAP, N1 latency, SP, CM, and DPOAE responses as a function of stimulus intensity. Shown are functions recorded after pre-drug artificial perilymph perfusion No. 2 (AP2) and after perfusion with increasing concentrations (3.3–100 μM) of ATP-γ-S. Data are represented as means ± S.E. across 5 animals. (From Kujawa et al., 1994b, with permission.)

RECEPTOR MECHANISMS

The action of these chemicals at the level of the OHC is summarized in Figure 2–15. The movement of the stereocilia opens the transduction channel allowing potassium ions into the cell which results in depolarization of the OHC (see review by Roberts, Howard, & Hudspeth, 1988). This activates the motor protein in the OHC membrane that causes the



1. transduction channel
2. motor
3. GABA–receptor & ion channel complex
4. ACh–receptor & ion channel complex
5. non–specific cation channel
6. ATP–receptor & ion channel complex
7. K_{Ca} and K_n channel
8. L–type Ca^{2+} channel

Figure 2–15. Model of an OHC illustrating the various ion channel proteins in the membrane and available to alter the membrane potential.

length change in the cell, the motor being sensitive to the voltage of the cell (depolarization or voltage change in a positive direction = decrease in length; hyperpolarization or voltage change in a negative direction = increase in length).

In Figure 2–15 I have represented three types of ion channels that will respond to voltage changes in the cell: a nonspecific cation channel, an L-type Ca^{2+} channel, and at least two types of Ca^{2+}-activated potassium channels which are represented in Figure 2–14 as one channel (Housley & Ashmore, 1992; Nakagawa et al., 1991). These ion channels are voltage-activated channels, that is, they will open in response to voltage changes and allow certain ions to move across the membrane of the OHC. The ion flows they allow will in turn change the voltage of the cell. GABA, ACh, and ATP, which are called "ligands," act on receptor proteins that regulate or open other ion channels and, therefore, these channels are called "ligand-gated channels." Ligand-gated channels are usually not opened by voltage changes. Ligands can act on the receptor protein which is an actual part of the ion channel or they can act on receptor proteins that interact with G proteins, which in turn can act on ion channels and other enzymes in the cell. In the latter case the mechanism of receptor action is called metabotropic and in the former case it is called ionotropic.

Whether the subdic receptor protein is metabotropic or ionotropic is currently being debated. As mentioned previously, Housley and Ashmore were the first to demonstrate that, in mammalian cochlear OHCs, ACh induces an opening of a channel (K_{ACh}) that allows K^+ ions to move across the membrane of the OHC, down its electrical and chemical gradient. At membrane potentials positive to –70 mV, K^+ will move out of the OHC and result in a hyperpolarization of the cell. Experimentally, the opening of the K_{ACh} channel induced by ACh requires the presence of both extracellular and intracellular free Ca^{2+}. Therefore, most researchers feel that the opening of the K_{ACh} channel by ACh involves an intermediate step which increases the level of free Ca^{2+} inside the cell adjacent to the K_{ACh} channel. This intermediate step may consist of ACh directly opening an ion channel linked to the receptor that allows Ca^{2+} to enter the cell, as illustrated in the Figure 2–15 (Fuchs & Murrow, 1992a). In contrast, Kakehata et al. (1993) suggest that ACh stimulates phosphatidylinositol metabolism via a PTX-sensitive G-protein. The phosphatidylinositol then releases Ca^{2+} from intracellular stores to act on the KACh channel. Such a mechanism is illustrated in Figure 2–16. So the major immediate question that needs to be answered is what is the mechanism of the subdic receptor: Does the receptor act by opening a Ca^{2+}-selective cation channel (ionotropic) or does it activate a G protein (metabotropic)?

In the OHCs, ATP appears to open a cation selective channel to allow sodium and Ca^{2+} into the cell (depolarization) as shown in Figure 2–15.

Others suggest that ATP may also activate the G-protein coupled to the phosphoinositol cascade in a manner similar to ACh, as shown in Figure 2–16 (Niedzielski & Schacht, 1992).

GABA appears to open an ion channel permeable to the chloride ion. This would hyperpolarize the cell similar to the effects of acetylcholine.

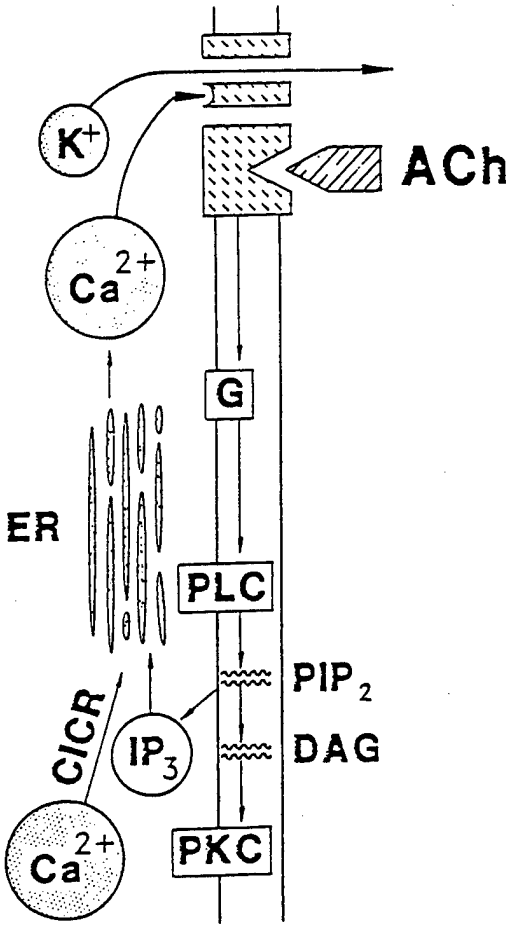


Figure 2–16. An alternative model (metabotropic) of the ACh receptor\ion channel complex illustrating a possible role for G protein and ionositol triphosphate (IP_3) in providing the intracellular Ca^{2+} for opening the K^+ channel. G: g protein; PLC: phospholipase C; PIP_2: phosphoinositol bisphosphate; DAG: diacylglycerol; PKC: phosphokinase C; CICR: calcium-induced calcium release; ER: endoplasmic reticulum.

In summary, these voltage-sensitive and ligand-activated channels in concert or alone can modify the voltage of the cell and the cells' length and length-change response to sound. In this manner, the active process is regulated and modulated. When and how all these channels are orchestrated to yield a functional active process awaits additional research.

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GLOSSARY

Autocrine. A chemical substance, also known as messenger, that is released from cells and reaches targets (receptors) located on the same cell by diffusion, for example, autoneurotransmitters.

Depolarization. Change in the resting membrane potential of a cell in the positive direction, less negative.

Hyperpolarization. Change in the resting membrane potential of a cell in the negative direction, less positive.

Ionotropic. A receptor mechanism for a neurotransmitter: the receptor protein is part of an ion channel\receptor protein complex and opens or closes the ion channel directly in response to the presence of a ligand (i.e., any compound that binds to a receptor) for that receptor.

Metabotropic. A receptor mechanism for a neurotransmitter: the receptor protein acts via another protein such as a g protein to activate or inhibit enzymes and possibly open, close, or modify ion channels.

Muscarinic receptor protein. The receptor activated by the neurotransmitter acetylcholine (ACh), and the drug, muscarine, located at the autonomic innervation of glands, cardiac muscle, and smooth muscle—abbreviated M.

Neurotransmitter. Chemical released from a sensory receptor cell or nerve cell on depolarization of that cell. On release the chemical diffuses across the gap between the releasing cell and an adjoining cell to act on the adjoining cell to induce a change in its electrical or chemical properties.

Nicotinic receptor protein. The receptor activated by the neurotransmitter acetylcholine (ACh), and by the drug, nicotine, located at the neuromuscular junction of skeletal muscle—abbreviated Nm.

Paracrine. A chemical substance, also known as a messenger, that is released from cells and reaches different target cells by diffusion, for example, neurotransmitters.

Receptor protein. Protein in the membrane of a cell that accepts a chemical messenger (ligand) such as the neurotransmitter, changes its configuration on accepting the neurotransmitter, and so induces subsequent reactions in the cell, such as opening of an ion channel to allow for diffusion of that ion down its concentration and electrical gradient.

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APPENDIX # 5
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HEARING RESEARCH

Chronic low-level noise exposure alters distortion product otoacoustic emissions

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Abstract

Chen et al. (1995) recently reported an altered response to the application of ATP in outer hair cells (OHC) isolated from guinea pigs continuously exposed for 10 or 11 days to a 65 dB SPL (A-scale) narrow-band noise (1.1–2.0 kHz). The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen et al. (1995) alters cochlear function. Cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs, as well as, the amount of contralateral suppression of DPOAE amplitudes were chosen for study. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles. The animals had either been exposed to the low-level noise for 3 or 11 days or not exposed at all ($n = 13$ animals per group). Results demonstrate that this noise induces frequency-dependent and very localized reductions in $2f_1$-f_2 DPOAE input/output (I/O) functions. However, the f_2-f_1 DPOAE I/O functions appear to be insensitive to the noise exposure. No noise-related changes were found in the amount of contralateral suppression between the different exposure groups, with the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced. The $2f_1$-f_2 DPOAE amplitude alterations lend support to the conclusions of Chen et al. (1995) that chronic low-level noise exposure induces molecular changes in the OHCs which may, in turn, alter cochlear function.

Keywords: Noise exposure; Distortion product otoacoustic emission; Outer hair cell

1. Introduction

Sound exposure induces changes in the structure and function of the auditory pathway (Saunders et al., 1985, 1991). These changes depend on the duration, intensity, and temporal pattern of the exposure. Continuous sound exposure produces more profound effects on the auditory system than interrupted sound exposure of equal acoustic energy (Ward, 1976, 1991; Bohne et al., 1985, 1987; Clark et al., 1987; Clark, 1991; Fredelius and Wersäll, 1992). The alterations in auditory function after very intense levels of sound can be attributed to mechanical changes in cochlear structure. On the other hand, low levels of sound produce complex changes within the cochlea that are not well described.

Above a certain level, continuous noise exposure will result in a decrease in auditory sensitivity. This decrease in sensitivity becomes larger during the first 24 h of exposure and then plateaus as the exposure continues, remaining stable during exposures lasting as long as 3 years (Clark and Bohne, 1987). This pattern of sensitivity change is referred to as an asymptotic threshold shift (ATS). ATS has been demonstrated in chinchillas (Carder and Miller, 1972; Mills, 1973, 1976; Bohne and Clark, 1982; Clark and Bohne, 1987), monkeys (Moody et al., 1976), guinea pigs (Syka and Popelar, 1980), and humans (Melnick, 1976). The level and duration of noise exposure determines whether ATS will fully recover (temporary threshold shift, TTS) or have a permanent component (permanent threshold shift, PTS; see Fig. 1 in Drescher, 1976).

The minimum level of continuous noise exposure required to produce a threshold shift depends upon the band of noise used and the species of animal under investigation. These minimum levels have been fairly well worked out in chinchillas for octave bands of noise centered at 0.5 and 4.0 kHz. Carder and Miller (1972) suggested that a 0.5 kHz octave band noise should not induce an elevation of auditory threshold when the exposure level is at or below 65 dB SPL. This was supported by Bohne (1976) who observed no structural damage to chinchilla hair cells after

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9 days of exposure to a noise of 65 dB SPL and the same spectral characteristics. For an octave band of noise centered at 4.0 kHz, Mills (1973) suggested that a level at or below 47 dB SPL should not produce ATS. According to these results, as the center frequency of the noise band increases, the exposure level required to produce ATS decreases.

To date, there is little, if any, information about the minimum level of continuous noise required to produce a threshold shift in guinea pigs. Davis et al. (1935) exposed nine guinea pigs to a 600 Hz pure tone at 65 dB for 70 days and found slight losses in sensitivity in only one of the animals. Canlon and Fransson (1995) exposed guinea pigs to a 1.0 kHz pure tone at 81 dB SPL for 24 days. They reported that this exposure did not cause any significant changes in auditory brainstem response thresholds and DPOAE amplitudes of the noise-exposed animals as compared to the unexposed animals. Syka and Popelar (1980) demonstrated the existence of an ATS over the course of a 5 day exposure to a 100 dB SPL third octave band of noise centered at 2.0 kHz. However, the authors did not explore the minimum level of noise necessary because a significant PTS was still present 120 days post-exposure.

Chen et al. (1995) reported that a 10 or 11 day exposure to a 65 dB SPL (A-scale) narrow-band noise (1.1–2.0 kHz) altered the response of guinea pig isolated OHCs to ATP application. This level of noise is very close to the minimum levels suggested to induce changes that would be observed as a threshold shift in chinchillas exposed to an octave band noise centered at 0.5 kHz (Carder and Miller, 1972). Thus, it is possible that the particular noise used by Chen et al. (1995) changes auditory threshold. To partially address this question, we asked whether the noise used by Chen et al. changes cochlear function? Puel et al. (1988, 1995) demonstrated that a low level of intense sound affects the active process before it affects the passive process. Thus there is a high probability that since the noise used by Chen et al. was very low, it only affected the active process. Others, utilizing salicylate, have demonstrated that the active process is integrally linked to OHC function and low level cochlear mechanics (Puel et al., 1989; Shehata et al., 1991; Kujawa et al., 1992). Therefore, to answer the question raised by the study of Chen et al., we examined the effects of the chronic low-level noise exposure on the cubic DPOAE ($2f_1$-f_2) and the quadratic DPOAE (f_2-f_1). DPOAE measurements were chosen for study since they are believed to reflect the status of OHC function (Siegel and Kim, 1982; Siegel et al., 1982). Others have speculated that ATP may be released by the efferents synapsing on the OHCs (e.g., Eybalin, 1993), therefore, the response reported by Chen et al. may be detected as an alteration in the effectiveness of efferent

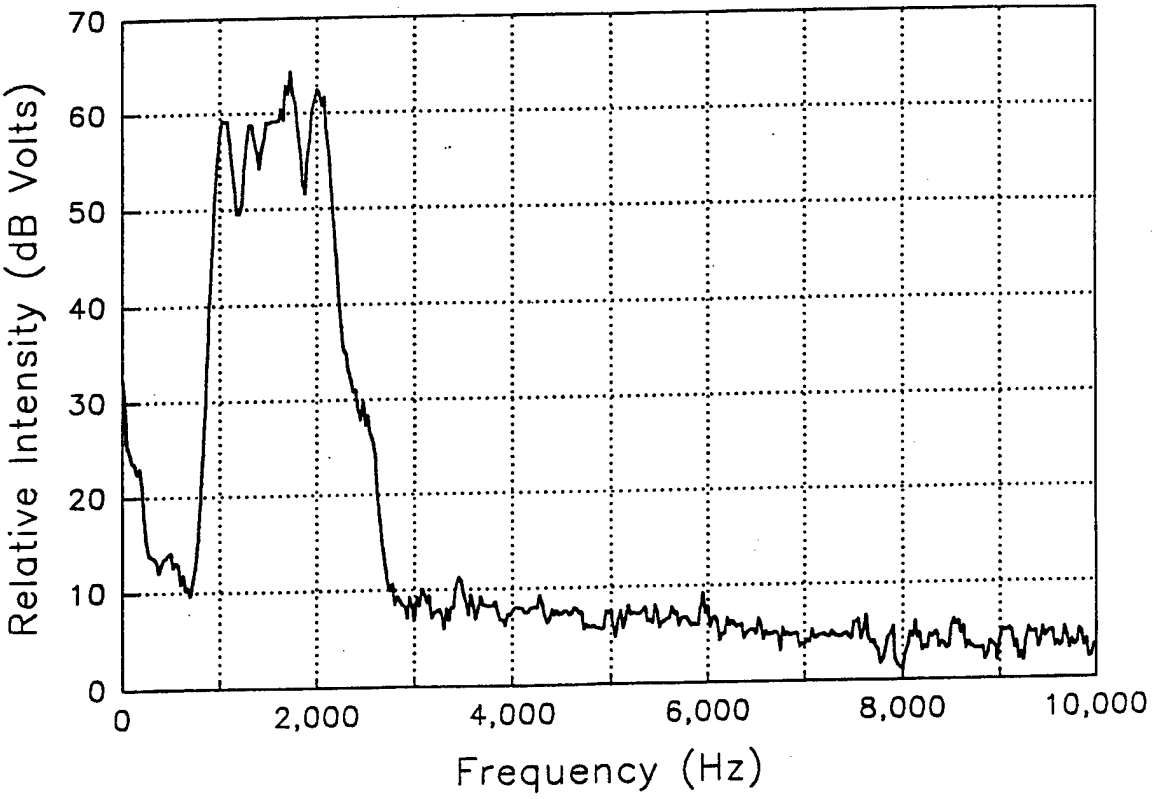

Fig. 1. The frequency spectrum of the 65 dB SPL (A-scale) noise used for continuous exposure. The sound level was monitored with a sound level meter and a 1\2 in. microphone (Brüel & Kjær type 2230 meter and type 4155 microphone) and viewed with a dynamic signal analyzer (Hewlett-Packard 3561A) set at a bandwidth of 95.485 Hz.

neurons. This was monitored by the amount of contralateral suppression of the DPOAEs (Puel and Rebillard, 1990; Kirk and Johnstone, 1993; Kujawa et al., 1993).

2. Methods

2.1. Subjects

Pigmented guinea pigs ($n = 39$) of either sex (300–500 g) were used as subjects. Upon delivery from the supplier, animals were randomly assigned to three groups ($n = 13$ per group) according to the number of days exposed to a low-level continuous noise (0, 3, and 11 days). The control group was not exposed to the noise. These animals were housed at the university's animal care facility. The experimental groups were exposed to a 65 dB SPL (A-scale) narrow-band noise (cutoff frequencies 1.1 and 2.0 kHz) 24 h/day for 3 or 11 days. These animals were tested approximately 1.5 h after removal from the noise because of the time necessary for the surgical procedures described below. All animals were given free access to food and water. The care and use of the animals that were used in this study were approved by LSUMC's Institutional Animal Care and Use Committee.

2.2. Noise exposure facility and noise generation

Unanesthetized guinea pigs were exposed to the continuous noise in groups of 10 or less in a small sound-attenuated booth. A small light was installed within the booth and controlled with a timer to provide the animals 12 h of light and 12 h of darkness.

The noise was generated by a WG2 Waveform Generator (Tucker-Davis Technologies) which was set to produce a uniform noise signal. This signal was filtered using a Brickwall Filter (Wavetek/Rockland Model 753A) configured in the bandpass mode with a low-frequency cutoff at 1.1 kHz and a high-frequency cutoff at 2.0 kHz. The level of the noise was controlled by a PA4 Programmable Attenuator (Tucker-Davis Technologies). Additional power was gained using a power amplifier (McIntosh MC2100) producing the final signal delivered to the speaker (Realistic 40-1286C; 8 Ω, 30 W).

Noise levels were monitored by placing a sound level meter (Brüel & Kjær Type 2230 Precision Integrating Sound Level Meter) within the booth. The microphone was positioned so that it was approximately at the level of the guinea pigs' ears and was placed at various positions around the booth to ensure that the noise was equally distributed throughout the booth. The spectrum of the noise as measured with a signal analyzer (Hewlett-Packard 3561A) is shown in Fig. 1.

2.3. General surgical methods

Immediately prior to testing, the animals were anesthetized by administering a dose of urethane (Sigma; 1.5 g/kg, i.p.), tracheotomized, and allowed to breathe unassisted. Supplementary doses of anesthetic (urethane, 1.5 g/kg) were given to maintain an adequate depth of anesthesia. Electrocardiogram and rectal temperature were monitored throughout each experiment and temperature maintained at 38 ± 1°C using a heating pad.

The surgical procedures used were similar to those described by Kujawa et al. (1993). Briefly, cartilaginous ear canals were exposed and partially removed to allow for proper placement of hollow ear bars which help secure the animal in the headholder. This procedure also ensured optimal coupling of the sound delivery system to both ears. Using a ventrolateral approach, the ipsilateral (right) auditory bulla was exposed and opened to gain access to the tendons of the middle ear muscles. These tendons were sectioned in all animals to prevent the involvement of middle ear muscle contraction on DPOAE measurements.

2.4. DPOAE generation and measurement

Equilevel primary tones, f_1 and f_2, were generated under computer control using Tucker-Davis System 2 audio processing equipment. More specifically, the computer generated primaries were sent to two separate channels of a DA1 digital-to-analog converter and attenuated to desired levels using PA4 programmable attenuators. The analog signals were then sent to two separate channels of an FT5 anti-aliasing low-pass filter with a 20 kHz cutoff frequency and then to an HB5 headphone buffer before being sent to two separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled to the right ear of each animal. DPOAEs were detected by a sensitive microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072). A dynamic signal analyzer (Hewlett-Packard, 3561A) was used to average the DPOAE responses for FFT analysis and spectral display (CF = DPOAE frequency; span = 1 kHz; BW = 3.75 Hz).

DPOAE amplitudes were measured for several combinations of f_1 and f_2 having a ratio $f_2/f_1 = 1.2$ (Table 1). This ratio is within the range describing the optimal frequency separation of f_1 and f_2 for the guinea pig (1.2–1.3; Brown, 1987; Brown and Gaskill, 1990). Since the place of origin of the DPOAEs is believed to be near, or at, the f_2 place on the cochlear partition (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993), all data are expressed as a function of f_2 rather than the frequency of the DPOAE (Table 1). DPOAEs were elicited with equilevel primaries tones ($L_1 = L_2$). Both quadratic (f_2-f_1) and cubic ($2f_1$-f_2) DPOAEs were studied. The primary tones were presented in descending order, starting at a level of 70 dB SPL and decreasing in 5 dB steps to 20 dB

Table 1
Primary tones (f_1,f_2) and corresponding cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs (in kHz)

f_1	f_2	f_2-f_1	$2f_1$-f_2
1.250	1.500	–	1.000
1.563	1.875	0.312	1.250
2.500	3.000	0.500	2.000
3.750	4.500	0.750	3.000
5.000	6.000	1.000	4.000
6.250	7.500	1.250	5.000
7.500	9.000	1.500	6.000
10.000	12.000	2.000	8.000

SPL. DPOAE I/O functions (i.e., plots of primary level vs. DPOAE amplitude for each f_2) were generated.

2.5. Contralateral noise generation

The 70 dB SPL wide-band noise delivered to the contralateral (left) ear to activate the efferents was also generated under computer control using Tucker-Davis System 2 audio processing equipment. In particular, a W1 Waveform Generator was set up to produce a uniformly distributed noise signal. The level of the was controlled with a PA4 Programmable Attenuator and then sent to a speaker (Etymotic Research, ER-2). A polyethylene tube (1.35 mm ID, 292 mm length) attached to the output of the speaker was used to deliver the noise down the hollow ear bar coupled to the entrance of the bony canal of the left ear. The spectrum of the noise presented to the contralateral ear was flat (±10 dB) from 0.9 to 15.8 kHz and rolled off

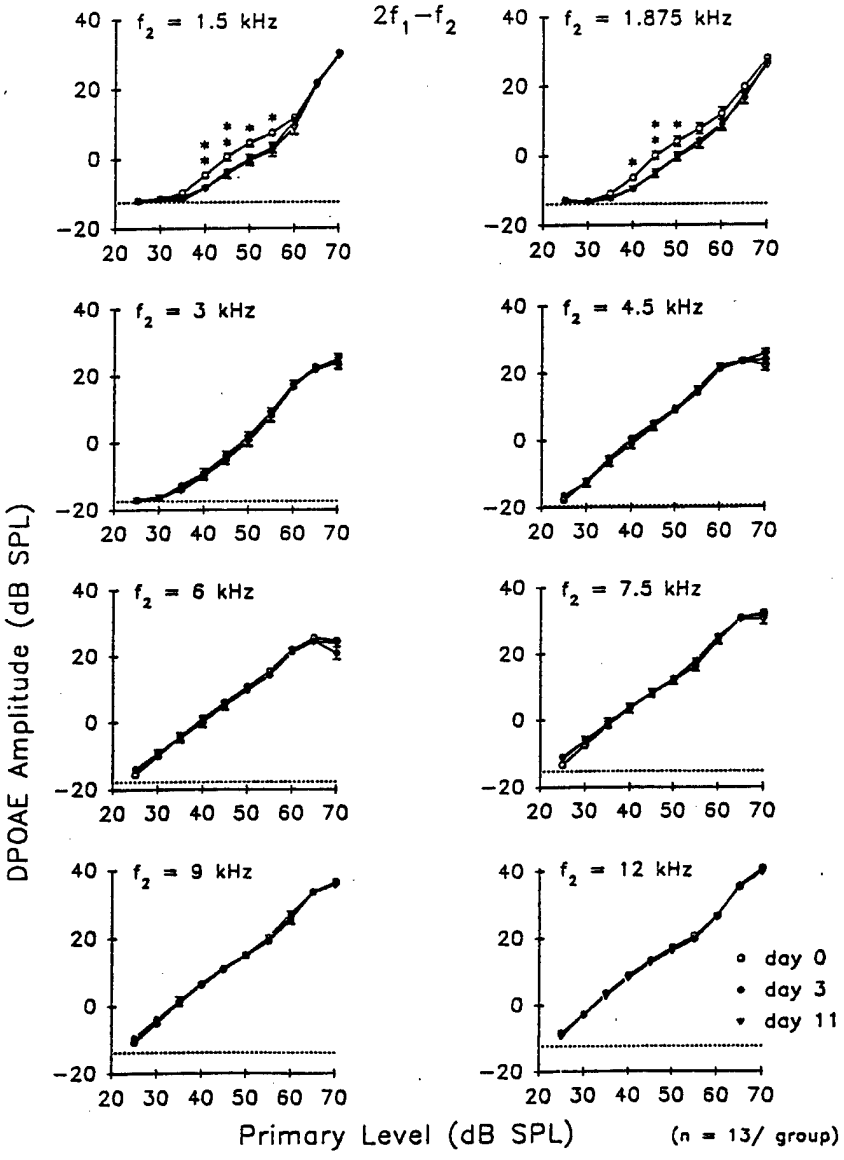

Fig. 2. Effect of continuous low-level noise exposure on $2f_1$-f_2 DPOAE I/O functions for different frequencies of f_2. Shown are data (means ± SE; n = 13 animals/group) for the unexposed group (day 0; ○), the group of animals exposed for 3 days (day 3; ●), and the group of animals exposed for 11 days (day 11; △). $^*P < 0.05$ and $^{**}P < 0.01$. The dashed line represents the noise floor.

approximately 50 dB/octave above 15.8 kHz.

2.6. Contralateral suppression measurements

DPOAEs were recorded, both with and without the wide-band noise (70 dB SPL) presented to the contralateral ear for several combinations of f_1 and f_2 ($f_2/f_1 = 1.2$; $L_1 = L_2 = 60$ dB SPL). Table 1 lists the primary frequencies and the corresponding DPOAEs (f_2-f_1 and $2f_1$-f_2) that were studied. The paradigm used to measure the amount of contralateral suppression is the same as that used by Kujawa et al. (1995). Briefly, five consecutive averages of DPOAE amplitude (10 spectra per averages) were first obtained in the absence of contralateral stimulation. The wide-band noise was then delivered to the contralateral ear and again five consecutive 10-spectra averages of the DPOAE amplitude were obtained. The final five consecutive averages were taken after the contralateral stimulation was removed. Each of these averages required approximately 5 s to complete. A total of 25 s of amplitude monitoring was obtained for each condition.

2.7. Data analysis

Data are presented as means ± SE. Effects of the low-level noise exposure on DPOAE I/O functions and contralateral suppression were quantified using 1-way (by exposure group) analysis of variance (ANOVA) and Newman-Keuls post-hoc tests. P values less than 0.05 were considered statistically significant.

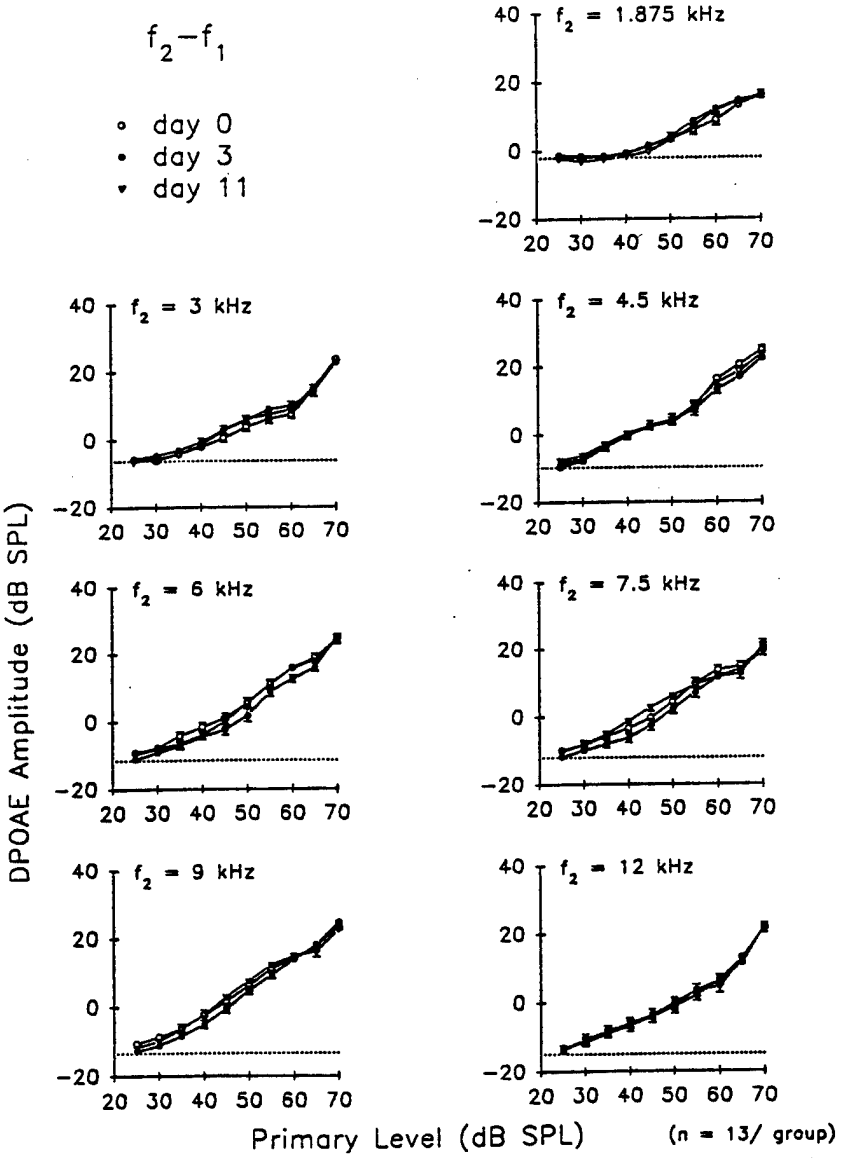

Fig. 3. Effect of continuous low-level noise exposure on f_2-f_1 DPOAE I/O functions for different frequencies of f_2. Shown are data (means ± SE; $n = 13$ animals/group) for the unexposed group (day 0; ○), the group of animals exposed for 3 days (day 3; ●), and the group of animals exposed for 11 days (day 11; △). The dashed line represents the noise floor.

3. Results

3.1. Effect of continuous low-level noise exposure on DPOAE I/O functions

Fig. 2 illustrates the $2f_1$-f_2 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL narrow-band noise for either 3 or 11 days. The results show significant differences between the control group and both noise-exposed groups in the DPOAE I/O functions elicited when f_2 was in the region of the noise band (Fig. 2; $f_2 = 1.5$ kHz and 1.875 kHz). Only intensity levels above the noise floor and ranging from 40 to 55 dB SPL were significantly different (Fig. 2). All other DPOAE I/O functions were not significantly different (Fig. 2; $f_2 = 3.0$, 4.5, 6.0, 7.5,

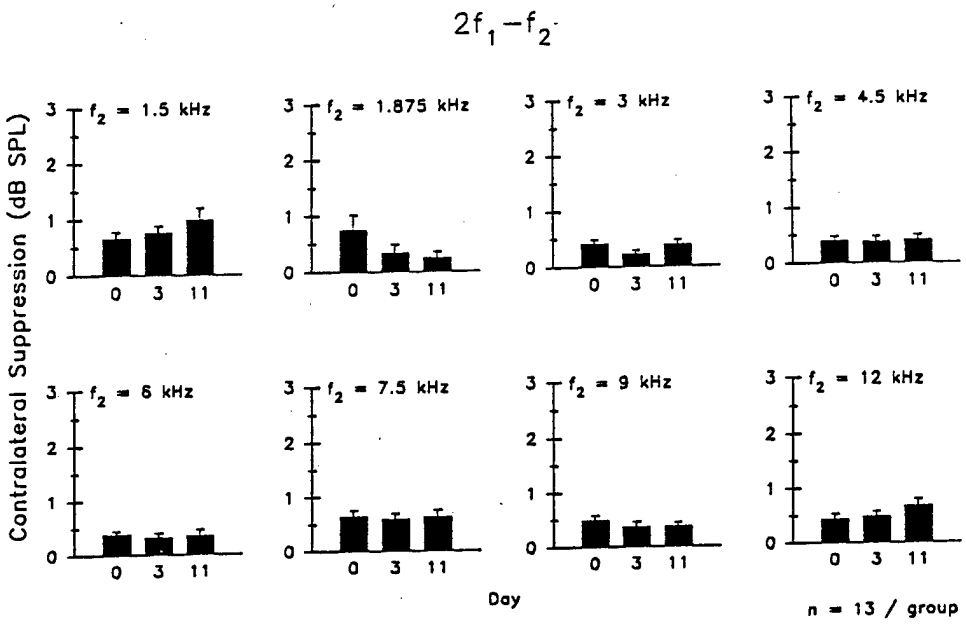

Fig. 4. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the $2f_1$-f_2 DPOAE for different frequencies of f_2. DPOAEs were monitored before (5 trials) and during (5 trials) presentation of wideband noise (70 dB SPL) to the contralateral ear. Each trial represents a 10 spectra average and required 5 s to complete. The bars represent the amount of contralateral suppression which was calculated by subtraction the mean of the 5 during trials from the mean of the 5 before trials. Data are represented as means ± SE ($n = 13$ animals/group).

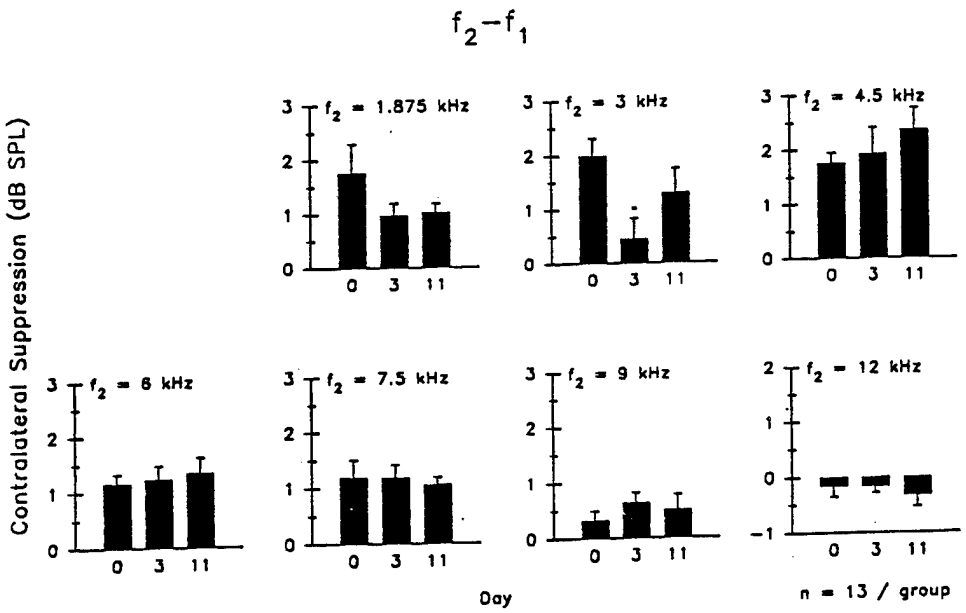

Fig. 5. Effect of continuous low-level noise exposure on the amount of contralateral suppression for the f_2-f_1 DPOAE for different frequencies of f_2. See Fig. 4 for additional information.

9.0, and 12.0 kHz; $P > 0.05$).

Fig. 3 illustrates the f_2-f_1 DPOAE I/O functions of the unexposed (control) guinea pigs and the guinea pigs exposed continuously to the 65 dB SPL narrow-band noise for either 3 or 11 days. The results did not show any significant differences in the DPOAE I/O functions between any of the groups tested for any combination of primary tones studied ($P > 0.05$).

3.2. Effect of continuous low-level noise exposure on contralateral suppression

Figs. 4 and 5 illustrate the average ($n = 13$ animals/group) amount of contralateral suppression measured in the unexposed group of animals and the groups of animals exposed to the 65 dB SPL narrow-band noise for both the $2f_1$-f_2 and f_2-f_1 DPOAE. The only suppression that was statistically different from day 0 was the 0.5 kHz f_2-f_1 DPOAE ($f_2 = 3$ kHz) at day 3 (Fig. 5). Other than for this one measure, there were no other statistically significant changes in the amount of contralateral suppression over the course of the continuous noise exposure in either the f_2-f_1 or $2f_1$-f_2 DPOAEs.

4. Discussion

4.1. Level of noise exposure and threshold

Chen et al. (1995) reported that a 65 dB SPL narrow-band noise (1.1–2.0 kHz) altered the response of OHCs to ATP. The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen et al. alters cochlear function. Results demonstrate that this noise induces a frequency-dependent and very localized reduction in $2f_1$-f_2 DPOAE I/O functions in guinea pigs exposed continuously for 3 and 11 days.

To the best of our knowledge, the level used in the present study is the lowest level of noise found to produce changes in the auditory function of the guinea pig. It is interesting to note that there were similar reductions in DPOAE amplitude for the groups of animals exposed for 3 and 11 days. This pattern of amplitude change resembles that of an ATS (Carder and Miller, 1972). There are no previous reports of 'ATS-like' changes in DPOAE amplitude in response to long-term noise exposure. However, we only monitored the changes in DPOAE amplitude on 2 days during our noise exposure protocol. Therefore, more experiments will have to be performed to characterize the time course of DPOAE amplitude change and to determine the degree of PTS and TTS.

4.2. Noise-induced effects on OHCs and/or cochlear mechanics

Significant amplitude reductions of the $2f_1$-f_2 DPOAE occurred only when the frequency of f_2 was within the noise exposure band. This finding is consistent with the belief that the f_2 place along the cochlear partition is the generation site of $2f_1$-f_2 DPOAEs (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993; Puel et al., 1995). Damage to this place results in the reduction and possibly complete loss of the corresponding $2f_1$-f_2 DPOAE (Siegel and Kim, 1982; Siegel et al., 1982; Zurek et al., 1982). Further, the changes in DPOAE amplitude induced by the noise exposure occurred only in the region of the I/O functions elicited by low-to-moderate level primaries. These low-level DPOAEs are thought to be mediated by active cochlear mechanics and are physiologically vulnerable to the same factors that normally damage or destroy OHC function.

We examined the effect of the noise on the f_2-f_1 DPOAE, since it is thought that these events reflect a different mechanical process in the cochlea then does the $2f_1$-f_2 DPOAE (Brown, 1988). Results show that there was no significant change in the amplitude of the f_2-f_1 DPOAE I/O functions even when the f_2 frequency was within the noise band. These results appear to indicate that the f_2-f_1 DPOAE are insensitive to the noise exposure used in our study.

4.3. ATP and efferents

Although speculative, it has been suggested that the cochlear efferents may release ATP onto the OHCs (Nakagawa et al., 1990; Eybalin, 1993). However, to date no response observed upon efferent activation has been attributed to released ATP. Nevertheless, it is possible that the reduction of the ATP response in the isolated OHCs reported by Chen et al. (1995) may decrease the effectiveness of the efferent innervation on the OHCs. Therefore, we examined effects of the noise exposure on the amount of DPOAE amplitude suppression obtained during presentation of sound to the contralateral ear, an effect known to be efferent-mediated (Warren and Liberman, 1989; Puel and Rebillard, 1990; Kirk and Johnstone, 1993; Kujawa et al., 1993). With the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced, no additional changes were found in the amount of contralateral suppression. This failure to detect a change may be due to the fact that the ATP may not play a role in this efferent phenomena.

4.4. Summary

In summary, results show that the chronic noise exposure used in this study and by Chen et al. (1995; 65 dB SPL, 1.1–2.0 kHz) does, in fact, alter cochlear function as measured by changes in the $2f_1$-f_2 DPOAE. The amplitude reductions were frequency-specific, occurring only in DPOAEs elicited when f_2 was within the noise exposure band. This level of noise (for this particular noise spec-

trum) probably approximates the minimum level of continuous noise required to induce a threshold shift in guinea pigs. In addition, the DPOAE amplitude alterations demonstrated here lend support to the conclusions of Chen et al. (1995) that chronic low-level noise exposure induces molecular changes in the OHCs which may, in turn, alter cochlear function.

Acknowledgements

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APPENDIX # 6
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Bobbin and Berlin

In Preparation for Hearing Research
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"Conditioning" the auditory system with continuous vs. interrupted noise of equal acoustic energy: Is either exposure more protective?

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Abstract

Prior exposure to moderate-level acoustic stimulation (conditioning) can reduce and/or prevent the deleterious effects of subsequent higher level exposures (Canlon et al., 1988; Campo et al., 1991). Both continuous and interrupted schedules of moderate-level noise have been used as conditioning exposures, and both schedules have been effective in providing protection against subsequent noise trauma. However, there is evidence to suggest that continuous noise exposures are more damaging to the cochlea than interrupted exposures of equal acoustic energy (Bohne et al., 1985, 1987), and moderate-level continuous and interrupted noise exposures differ in the pattern of auditory sensitivity change that they produce over time (Carder and Miller, 1972; Miller et al., 1963). A question arises as to whether there are differences in the amount of protection afforded by prior conditioning of the auditory system with moderate-level continuous or interrupted noise.

The purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous vs. interrupted, moderate-level noise. Differences were determined by monitoring the changes that occurred in cubic ($2f_1$-f_2) distortion product otoacoustic emission (DPOAE) amplitude growth functions subsequent to a traumatizing noise exposure (105 dB SPL, 1.0-2.0 kHz octave band noise presented 24 hours per day for 3 days) in guinea pigs which had been conditioned with either continuous (89 dB SPL, 1.0-2.0 kHz octave band noise presented 24 hours per day for 11 days) or interrupted noise (95 dB SPL, 1.0-2.0 kHz octave band noise presented on a 6 hours "on"/18 hours "off" schedule for 11 days) of equal acoustic energy.

Results suggest that there are significant differences in the degree of protection provided by prior sound conditioning with the continuous and interrupted schedules of moderate-level noise used in this study. Specifically, the interrupted conditioning protocol appears to afford some degree of protection against the damaging effects of the traumatizing noise exposure. However, the frequency region that is protected is limited to frequencies above the noise exposure band. Conversely, there is a lack of any consistent and sizable protective effect found across the entire test frequency range for the continuous sound conditioning protocol.

1. Introduction

Not all individuals exposed to similar noise conditions incur equal damage or hearing loss. There are several factors, both nonauditory and auditory, that have been suggested as influences on an individual's susceptibility to noise-induced hearing loss. Nonauditory factors include age (Szanto and Ionescu, 1983; Mills, 1992), gender (Szanto and Ionescu, 1983), eye color (Carlin and McCrosky, 1980; Carter, 1980; Kleinstein et al., 1984; Barrenäs and Lindgren, 1990; Barrenäs and Hellström, 1996), and smoking history (Barone et al., 1987; Prince and Matanoski, 1991). Also, the use of ototoxic drugs (McFadden and Plattsmier, 1983; McFadden, 1986; Gratton et al., 1990) has been shown to exacerbate the effects of noise exposure. Auditory factors include the efficiency or attenuation characteristics of the acoustic reflex (Zakrisson et al., 1980; Borg and Nilsson, 1984; Rodriguez and Gerhardt, 1988;), activation of the medial cochlear efferent system (Cody and Johnstone, 1982; Puel et al., 1988b; Rajan, 1992), and an individual's previous noise exposure history (Miller et al., 1963; Clark et al., 1987).

In recent years, considerable attention has been given to the idea that susceptibility of the auditory system to noise-induced hearing loss may be lessened by an individual's previous history of noise exposure. Prior exposures to moderate-level acoustic stimulation (i.e., conditioning) can reduce (and in some instances prevent) the deleterious effects of subsequent higher level (and usually damaging) exposures (Canlon et al., 1988, 1992; Campo et al., 1991; Henderson et al., 1992; Ryan et al., 1994). Both continuous and interrupted schedules of moderate-level noise have been used as conditioning exposures, and both schedules have been effective in providing protection against subsequent noise trauma.

There is evidence to suggest that the temporal pattern of a noise exposure (i.e., continuous vs. interrupted) is an important factor which determines the magnitude of hair cell damage and the pattern of sensitivity changed produced by an exposure. Many investigations have focused on the histological changes associated with continuous and interrupted exposure schedules and have shown that continuous noise exposures are more damaging to the cochlea than interrupted exposures of equal acoustic energy (Bohne et al., 1985, 1987; Fredelius and Wersäll, 1992). Others have reported that moderate-level continuous and interrupted noise exposures differ in the pattern of auditory sensitivity change that they produce over time (Carder and Miller, 1972; Miller et al., 1963). Continuous noise exposure results in a decrease in sensitivity that grows over the first 24 hours of exposure and then stabilizes as the exposure continues. This pattern is referred to as an asymptotic threshold shift (Carder and Miller, 1972). Interrupted noise exposure, on the other hand, results in an initial decrease in sensitivity during the first few days of exposure, followed by a return towards pre-exposure levels on subsequent days of exposure (Miller et al., 1963). This pattern of change is known as "toughening".

It is currently unknown whether there are differences in the amount of protection afforded by prior conditioning of the auditory system with either moderate-level continuous or interrupted noise exposures of equal acoustic energy. Thus, the purpose of this study is to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous versus interrupted moderate-level noise. The differences in protective effect of the two conditioning schedules were determined by monitoring changes that occur in distortion product otoacoustic emission (DPOAE) amplitude growth functions after a subsequent higher level (traumatizing) exposure in guinea pigs (*Cavia cobaya*) that had been conditioned with either continuous or interrupted noise. Both conditioning exposure schedules had the same total acoustic energy consistent with the Equal Energy Hypothesis (EEH; Eldred et al., 1955). This equal energy requirement was considered important in the design of the study because it yielded noise exposures that differed only in their temporal pattern.

2. Methods

2.1. Subjects

Experiments were performed on 84 pigmented guinea pigs (*Cavia cobaya*) of either sex weighing between 500 and 850 grams. Upon delivery from the supplier, animals were randomly assigned to six groups (n = 14 per group). Group assignments were as follows:

Aged Normal Group (Unexposed) - The animals in this group were housed and cared for in the Animal Care facility for 7-8 weeks prior to being tested. The level of the ambient noise within this facility was usually between 40 and 50 dB SPL (A-scale). However, during a ½ hour period each day when the cages were being cleaned, this level could be as high as 80 to 90 dB SPL (A-scale).

Continuous Conditioning Group - The animals in this group were exposed continuously for 11 days to an 89 dB SPL (A-scale) octave band noise (1-2 kHz). These animals were tested within 2 hours after their removal from the noise exposure booth. This 2 hours represented the time needed for surgical manipulation (approximately 1½ hours) plus a ½ hour recovery period from the surgery.

Interrupted Conditioning Group - The animals in this group were exposed to a 95 dB SPL (A-scale) octave band noise (1-2 kHz) 6 hours per day for 11 days (6 hrs "on"/18 hrs "off"). These animals were tested within 2 hours after their removal from the noise exposure booth. This 2 hours represented the time needed for surgical manipulation (approximately 1½ hours) plus a ½ hour recovery period from the surgery.

Continuous Conditioning, then Blast Group - The animals in this group were exposed continuously for 11 days to an 89 dB SPL octave band noise (1-2 kHz), given 1 week to recover at Louisiana State University Medical Center's Animal Care facility, exposed continuously to a 105 dB SPL (A-scale, 1-2 kHz) for 3 days, and

then allowed to recover for 4 weeks in the Animal Care facility before being tested.

Interrupted Conditioning, then Blast Group - The animals in this group were exposed 6 hours per day for 11 days to a 95 dB SPL octave band noise (1-2 kHz; 6 hours "on"/18 hours "off"), given 1 week to recover at Louisiana State University Medical Center's Animal Care facility, exposed continuously to a 105 dB SPL (A-scale, 1-2 kHz) for 3 days, and then allowed to recover for 4 weeks in the Animal Care facility before being tested.

Control/Blast Only Group - The animals in this group were exposed only to the 105 dB SPL (A-scale, 1-2 kHz) for 3 days, and then allowed to recover for 4 weeks in the Animal Care facility before being tested. The amount of time these animals spent in the facility prior to exposure was such that the time required to condition the animals in the other groups (11 days) and allow them to recover (1 week) were equivalent.

During periods of noise exposure, the animals were housed in a small sound attenuating booth (See Section 2.2.1 for description). Unexposed animals, along with animals recovering from the noise exposure, were housed and cared for by Louisiana State University Medical Center's Animal Care facility, approved and certified by the American Association of Laboratory Animal Science.

Only animals with a normal Preyer reflex and no obvious outer or middle ear pathology were included in the study. All animals used in this study were treated in accordance with federal, state, and institutional guidelines and the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1985). The care and use of the animals were approved by the Medical Center's Institutional Animal Care and Use Committee.

2.2 Noise Generation and Exposure Methods

2.2.1 Noise exposure facility

During periods of noise exposure, unanesthetized guinea pigs were housed in groups of 10 or less in a small sound-attenuated booth (approx. interior dimensions 76 x 60 x 40 cm; Industrial Acoustics Company, Inc., Serial #101655) contained within a larger sound-attenuated booth (Industrial Acoustics Company, Inc., Serial #106138). The walls of the smaller booth were lined with hard, reflective surfaces to produce uniform sound levels throughout the chamber. The speaker was mounted on a wooden surface which covered the booth ceiling. The speaker was approximately 40 cm above the level of the guinea pigs' ears. A small light was also mounted on the wooden surface. The light was controlled by a timer which allowed for 12 hours of illumination and 12 hours of darkness. This sequence of light and dark periods provided the animals with a simulated diurnal cycle. Small holes were drilled into the wood (in irregular patterns) beneath the intake and exhaust vents to allow for proper air exchange within the booth. A small exhaust fan was mounted on the side of the booth for additional ventilation. The

floor of the booth was lined with a metal pan filled with animal bedding (Sani-Chips).

2.2.2 Noise generation and calibration procedures

Both the moderate-level conditioning noise and the traumatizing noise were generated by a WG2 Waveform Generator (Tucker-Davis Technologies) which was set in the "Uniform" mode. This signal was bandpass filtered using a Brickwall Filter (Wavetek/Rockland Model 753A) with a low frequency cutoff at 1.0 kHz, a high frequency cutoff at 2.0 kHz, and a roll-off of 115 dB/octave. The level of the filtered noise was controlled by a PA4 Programmable Attenuator (Tucker-Davis Technologies). Additional power was gained using a power amplifier (McIntosh MC2100) producing the final signal that was delivered to the speaker (Realistic 40-1286C; 8Ω, 30 watts).

The spectrum of the noise used in this study was an octave band noise with the low- and high-cutoff frequencies at 1.0 and 2.0 kHz, respectively. The continuous and interrupted schedules of the moderate-level conditioning noise had equal acoustic energy as defined by the EEH (Eldred et al., 1955). As mentioned earlier, the EEH implies that for every doubling of exposure duration, the intensity of the noise must be decreased by 3 dB for the total acoustic energy to remain constant. In this study, the continuous conditioning noise was presented at a level of 89 dB SPL (A-scale) for 24 hours per day for 11 days, while the interrupted conditioning noise was presented at a level of 95 dB SPL (A-scale) for 6 hours per day (6 hours "on"/18 hours "off") for 11 days. The high-level traumatizing noise was presented continuously for 3 days at 105 dB SPL (A-scale). These levels were chosen because they approximated the levels of conditioning and traumatizing noises used in previous studies concerned with this type of protection phenomenon (Canlon et al., 1988; Campo et al., 1991).

Noise levels were monitored daily using a ½ inch condenser microphone (Brüel & Kjaer Type 4133) and preamplifier combination that were connected to a measuring amplifier (Brüel & Kjaer Type 2610). This system was calibrated with a sound level calibrator (Brüel & Kjaer Type 4230) which produces a nominal sound pressure of 94 dB ± 0.3 dB (re: 20 μPa). The microphone was positioned so that it was approximately at the level of the guinea pigs' ears and was placed at various positions around the booth to ensure that the noise was equally distributed throughout the booth. Noise levels were found to vary ± 2 dB depending upon microphone position. The background level inside the booth with the noise off, doors closed, and animals present was approximately 40 dB SPL (A-scale). The voltage across the speaker was also checked daily using a digital voltmeter (Wavetek Corporation BI-DM15XL). The voltages corresponding to the levels of noise used in this study, i.e., 89, 95, and 105 dB SPL, were approximately 0.45, 0.90, and 2.9 volts (rms). The linearity of the speaker (and noise generation system) was verified by converting the ratios of these voltages to dB to make certain that this value coincided

with the amount of change (in dB) of the attenuator setting.

The spectrum of the noise was obtained by connecting the AC output of the measuring amplifier (Brüel & Kjaer Type 2610) to the input of a signal analyzer (Hewlett-Packard 3561A). The spectrum of the noise is shown in Figure 1 for each exposure level.

2.3 General surgical methods

Immediately prior to testing, the animals were anesthetized by administering a dose of urethane (ethyl carbamate, Sigma; 1.5 g/kg, i.p.), tracheotomized, and allowed to breathe unassisted. Supplementary doses of anesthetic (urethane, 1.5 g/kg) were given if necessary to maintain an adequate depth of anesthesia (as indicated by a lack of a withdrawal response to deep pressure and pain applied to the animal's paw). Electrocardiogram and rectal temperature were monitored throughout each experiment and temperature was maintained at 38 ± 1°C using a heating pad.

The surgical procedures used were similar to those described by Kujawa et al. (1992, 1993). Briefly, cartilaginous ear canals were exposed and partially removed to allow for proper placement of ear bars used to secure the animal in a modified Narishige stereotaxic headholder. This procedure also ensured optimal coupling of the sound delivery and response retrieval system to the test ear. Using a ventrolateral approach, the ipsilateral (right) auditory bulla was exposed and opened to gain access to the tendons of the middle ear muscles. These tendons were sectioned in all animals to prevent the involvement of middle ear muscle contraction on the DPOAE measurements. The surgical procedures required approximately 1½ hours to complete. All experiments were acute.

2.4 DPOAE generation and calibration procedures

Cubic ($2f_1$-f_2) DPOAEs were elicited by the presentation of equilevel (L_1=L_2) primary tones (f_1 and f_2) at various frequencies which yielded a f_2/f_1 ratio of 1.2. The primaries were generated under computer control using Tucker-Davis System 2 audio processing equipment. More specifically, the computer generated primaries were sent to two separate channels of a DA1 Digital-to-Analog Converter and attenuated to desired levels using PA4 Programmable Attenuators. The attenuated analog signals were then sent to two separate channels of an anti-aliasing low-pass filter with a 20 kHz cutoff frequency (FT5) and then to the HB5 Headphone Buffer before being sent to separate speakers (Etymotic Research, ER-2) housed within an acoustic probe assembly. The acoustic probe assembly was tightly coupled to the right ear of each animal. DPOAEs were then detected by a sensitive microphone (Etymotic Research, ER-10) also housed within the probe assembly and amplified using a microphone preamplifier (Etymotic Research, ER-1072). A dynamic signal analyzer (Hewlett-Packard, 3561A) was used to average the DPOAE responses for FFT analysis and spectral display (25 rms averages; center frequency = DPOAE frequency; span = 1 kHz; bandwidth = 3.75 Hz).

Calibration of the primary tones was performed at the outset of the study and then twice a week thereafter. This was accomplished by coupling the acoustic probe assembly to a ¼ inch condenser microphone (Brüel & Kjaer Type 4135) and then cross-checking the output of the speakers using (1) the ER-10 probe microphone and (2) the B&K condenser microphone. The signal from the probe microphone and preamplifier (ER-10 and ER-1072) was sent to the signal analyzer and the level of each of the primary tones was obtained from the spectral display (center frequency = primary tone frequency; span = 1 kHz; bandwidth = 3.75 Hz). For this system, the conversion of dBV to dB SPL was obtained by adding 120 to the dBV value at the peak of the corresponding primary frequency. The signal transduced by the condenser microphone and preamplifier was sent to the measuring amplifier (Brüel & Kjaer Type 2610) and the level of the primary tones was indicated by the deflection of the needle on the meter scale (measured in dB SPL). The equivalence of the primary levels using both measurement systems was verified at each calibration session.

2.5 DPOAE input-output measurements

Cubic ($2f_1$-f_2) DPOAEs were measured for several combinations of f_1 and f_2 (See Table 1). For the proposed experiments, the f_2/f_1 ratio was held constant at 1.2, which is within the range describing the optimal frequency separation of f_1 and f_2 for the guinea pig (1.2-1.3; Brown, 1987; Brown and Gaskill, 1990). This ratio has been used previously in our laboratory (Kujawa et al., 1992, 1993) and has been adopted as a laboratory standard for purposes of comparing old and new DPOAE data. In addition, because the place of the DPOAEs is believed to be near, or at, the f_2 place on the cochlear partition (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993; Puel et al., 1995), all data were expressed as a function of f_2 rather than the actual frequency of the DPOAE. These particular values of f_2 were chosen for study because their corresponding frequencies map out (in half octave steps) the frequency spectrum of the noise band used to expose the animals and the region of possible OHC loss along the cochlear partition.

DPOAE responses were elicited with equilevel primaries (L_1=L_2). The primary tones were presented in descending order, starting at a level of 70 dB SPL and decreasing in 5 dB steps to 20 dB SPL. The amplitudes of the DPOAEs, defined as the spectral peak corresponding to the $2f_1$-f_2 frequency (as viewed on the signal analyzer), were recorded manually in dBV from the FFT spectra (25 averages) and later converted to dB SPL (re: 20 μPa). Plots of primary level vs. DPOAE amplitude (amplitude growth functions) were generated.

2.6 Data management and analysis

Statistical analysis of the data was performed using SigmaStat® Statistical Software (Version 2.0 for Windows® 95, NT, & 3.1; Jandel Scientific Corporation). The response measurements

(DPOAE amplitude growth functions) of all exposure groups were analyzed using a between-group, three-way (exposure group x frequency x primary level) analysis of variance (ANOVA) procedure. The Tukey multiple comparisons (post hoc) test was performed when significant differences were found. P values less than 0.05 were considered statistically significant.

3. Results

3.1 Baseline DPOAE responses

DPOAE amplitude growth functions in aged normal guinea pigs was first performed to establish the baseline responses for the DPOAE parameters (e.g., f_2/f_1 ratio and f_2 test frequencies) used in this study. The mean DPOAE amplitude growth functions obtained from the Aged Normal Group (Figure 2a-i) were relatively consistent with the normative DPOAE results of other studies that used guinea pigs as experimental subjects (Brown, 1987; Brown and Gaskill, 1990). The DPOAE amplitudes were generally 30-50 dB SPL below the level of the primary tones at each of the intensity levels and each frequency tested. Although there were instances where the amplitude growth functions were highly nonmonotonic (e.g., f_2 = 707- and 1000 Hz), the overall growth rate of the distortion product amplitudes was approximately linear (i.e., a 1 dB increase in DPOAE amplitude for every 1 dB increase in primary level).

The variability of the amplitude growth functions across animals was small at each frequency tested, with standard deviations of usually less than 3 dB. However, there was a tendency for increased variability for the lower frequency DPOAEs (f_2 = 707-, 1000-, and 1414 Hz), especially in the highly nonmonotonic regions of the amplitude growth functions (primary tones ranging from 45 to 65 dB SPL). In such cases, the standard deviations were no more than 6.5 dB. When the frequency of f_2 was 2828-, 4000-, and 5656 Hz, an increase in intersubject variability also occurred in the region of the DPOAE amplitude growth functions generated by high-level primary tones (60-70 dB SPL). In this intensity region, the growth functions of many guinea pigs within this group exhibited rollover; however, the standard deviations still did not exceed 5 dB.

3.2 Effects of sound conditioning on DPOAE responses

The DPOAE amplitude growth functions shown in Figure 2 (a-i) represent the average responses (mean ± S.E.; n=14) of the Continuous Conditioning Group and the Interrupted Conditioning Group. These are plotted in contrast with the average responses (mean ± S.E.; n=14) of the Aged Normal Group to demonstrate the effect of both sound conditioning protocols on normal DPOAE responses and to compare the effects of the two different schedules of conditioning noise.

All main effects (Group, Frequency, Intensity) and interactions of the statistical analysis were statistically significant ($P<0.001$). When the interactions between the factors were examined more thoroughly, it was found that (1) the effects

of the Group x Frequency interaction depended on the intensity level of the primaries; and (2) the effects of the Group x Intensity interaction depended on the frequency of f_2. There was not a significant Group x Frequency interaction when the intensity of the primary tones was 20 dB (P = 1.000); however, this interaction was significant at all other intensity levels. In addition, there were not significant Group x Intensity interactions when the frequency of f_2 was 8000 (P = 0.826) and 11312 Hz (P = 0.997), whereas significant interactions were found at all other f_2 frequencies.

3.2.1 Continuous sound conditioning

To better describe the effects of continuous sound conditioning on DPOAE responses, the differences in the amplitude growth functions between the Aged Normal Group and the Continuous Conditioning Group were obtained by subtracting the average DPOAE amplitudes of the sound conditioned group from the baseline DPOAE responses. Figure 3a provides a graphical representation of the mean amplitude differences between the two groups (in dB) plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The positive excursions from zero represent the magnitude of the amplitude reductions induced by the noise exposure, whereas the negative excursions from zero show where the magnitude of the DPOAE responses were actually larger in the exposed group than in the unexposed controls.

The continuous conditioning exposure caused reductions in DPOAE amplitudes in a frequency- and intensity-dependent manner. The overall bell shape of Figure 3a demonstrates the frequency-dependence of the noise-induced effects on the DPOAE responses. The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and higher frequencies. Specifically, the greatest amplitude reductions (approximately 12-18 dB) occurred in the frequency range spanning f_2 frequencies of 1414-4000 Hz. This frequency region corresponds to the center and upper cutoff frequency of the noise exposure band used in this study (octave band noise; 1.0-2.0 kHz), and extends to one octave above the upper cutoff frequency. Smaller reductions (approximately 5-8 dB) were found at f_2 frequencies of 707-, 1000- and 5656 Hz, while at 8000- and 11312 Hz, the magnitude of the amplitude reductions was only 1-3 dB.

The pattern of the individual bars plotted at each frequency illustrates the intensity-dependence of the noise-induced effects on the DPOAE responses. In most cases, the greatest decreases in amplitude occurred for primary levels ranging from 40-60 dB SPL. At the lower f_2 frequencies (707- and 1000 Hz), there were actually places within the amplitude growth functions where the responses of the sound conditioned group were better or not much different than those of the normal control group. This occurred for primary levels ranging from 50-60 dB SPL. It is within this intensity range where a large dip occurred in the amplitude growth functions of the unexposed group of animals.

3.2.2 Interrupted sound conditioning

To better describe the effects of interrupted sound conditioning on DPOAE responses, the differences in the amplitude growth functions between the Aged Normal Group and the Interrupted Conditioning Group were obtained by subtracting the average DPOAE amplitudes of the sound conditioned group from the baseline DPOAE responses. Figure 3b provides a graphical representation of the mean amplitude differences between the two groups (in dB) plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The positive excursions from zero represent the magnitude of the amplitude reductions induced by the noise exposure, whereas the negative excursions from zero show where the magnitude of the DPOAE responses were actually larger in the exposed group than in the unexposed controls.

The interrupted conditioning exposure also caused reductions in DPOAE amplitudes in a frequency- and intensity-dependent manner. The overall "quasi" bell-shaped pattern of Figure 5 demonstrates the frequency-dependence of the noise-induced effects on the DPOAE responses. The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and virtually non-existent in the higher frequencies. For this exposure protocol however, the greatest amplitude reductions occurred in a narrower frequency range (relative to the continuous sound conditioning protocol), spanning f_2 frequencies of 2000-4000 Hz. The magnitude of the reductions within this range was approximately 11-16 dB. This frequency region corresponds to the upper cutoff frequency of the noise exposure band used in this study (octave band noise; 1.0-2.0 kHz) and extends to one octave above the upper cutoff frequency.

The pattern of the individual bars plotted at each f_2 frequency illustrates the intensity-dependence of the noise-induced effects on the DPOAE responses. In the frequency region where the interrupted noise exposure had its greatest effect (f_2 = 2000-4000 Hz), the largest decreases in amplitude occurred for primary levels ranging from about 40-60 dB SPL. At the lower f_2 frequencies (707- and 1000 Hz), there were actually intensity levels of the amplitude growth functions where the DPOAEs amplitudes recorded for the sound conditioned group were larger than those of the normal control group. These f_2 frequencies coincide with the lower cutoff frequency of the noise exposure band (1000 Hz) and a frequency ½ octave below it (707 Hz). These elevations in the DPOAE amplitudes relative to the normal controls occurred for primary levels ranging from 50-60 dB SPL. It is within this intensity range where a large dip occurred in the amplitude growth functions of the unexposed group of animals.

3.3 Effects of the traumatizing noise exposure on the DPOAE responses of unconditioned and sound conditioned animals

The DPOAE amplitude growth functions shown in Figure 4 (a-i) represent the average responses (mean ± S.E.; n=14) of the Blast

Only Group, the Continuous Conditioning then Blast Group, and the Interrupted Conditioning then Blast Group. These are plotted together to illustrate the effects of the traumatizing noise exposure in both unconditioned and sound conditioned animals.

3.3.1 Unconditioned animals

To show the effects of the traumatizing noise exposure on normal DPOAE responses, the differences in the amplitude growth functions between the Aged Normal Group and the Blast Only Group were obtained by subtracting the average DPOAE amplitudes of the group exposed to the traumatizing noise from the baseline DPOAE responses. Figure 5a provides a graphical representation of the mean amplitude differences between the two groups (in dB) plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The positive excursions from zero represent the magnitude of the amplitude reductions induced by the noise exposure, whereas the negative excursions from zero show where the magnitude of the DPOAE responses were actually larger in the exposed group than in the unexposed controls.

The overall shape of Figure 5a demonstrates the frequency-dependence of the noise-induced effects on the DPOAE responses caused by the high-level, traumatizing exposure. This pattern was different than the patterns of the noise-induced effects obtained from groups of animals exposed only to the sound conditioning noise (Figure 3a and 3b). The alterations in the DPOAE amplitude growth functions caused by this exposure were more widespread. The noise-induced reductions in DPOAE amplitudes were not only larger, but were also distributed across the entire test frequency range. The region of maximal effect was skewed toward the higher frequencies of f_2 tested. This region covered f_2 frequencies ranging from 2828-8000 Hz. The magnitudes of the DPOAE amplitude reductions within this frequency range were approximately 12-21 dB. Smaller reductions occurred for the lower frequencies of f_2 (707-2000 Hz). Still, the amplitude reductions within this low frequency range were quite large, with losses ranging from approximately 5-15 dB at intensity levels of the amplitude growth functions where the effects of the noise were maximal. The traumatizing noise had its least effect at the highest frequency of f_2 tested (f_2=11312 Hz), with reductions of only 2-4 dB.

The pattern of the individual bars plotted at each frequency illustrates the intensity-dependence of the noise-induced effects on the DPOAE responses. In the frequency region where the traumatic noise exposure had its greatest effect, the largest decreases in amplitude occurred for primary levels ranging from about 40-60 dB SPL. For the DPOAE amplitude growth functions measured at f_2 frequencies of 707-2828 Hz, the largest amplitude reductions occurred for the high level primary tones tested (65-70 dB SPL), suggesting that this high intensity noise also affected passive cochlear mechanics within this frequency range. At the lower f_2 frequencies (707- and 1000 Hz), there were actually intensity levels of the amplitude growth functions where

the responses of the sound conditioned group were better or not much different than those of the normal control group. This occurred for primary levels ranging from 50-60 dB SPL. It is within this intensity range where a large dip occurred in the amplitude growth functions of the unexposed group of animals.

3.3.2 Sound conditioned animals

3.3.2.1 Continuous sound conditioning

To show the effects of the continuous conditioning/traumatizing noise exposure combination on normal DPOAE responses, the differences in the amplitude growth functions between the Aged Normal Group and the Continuous Conditioning then Blast Group were obtained by subtracting the average DPOAE amplitudes of the group exposed to the traumatizing noise from the baseline DPOAE responses. Figure 5b provides a graphical representation of the mean amplitude differences between the two groups (in dB) plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The pattern of change of the DPOAE amplitude growth functions caused by the traumatizing noise exposure in animals previously sound conditioned on the continuous schedule of moderate-level noise was similar to that obtained for the unconditioned animals (Figure 5a). Although similar, there were differences in the DPOAE responses between the unconditioned (Blast Only) group and the sound conditioned (Continuous Conditioning then Blast) group. These differences are discussed in detail in Section 3.3.3.1 as they relate to the possible protective role of the continuous sound conditioning exposure.

3.3.2.1 Interrupted sound conditioning

To show the effects of the interrupted conditioning/traumatizing noise exposure combination on normal DPOAE responses, the differences in the amplitude growth functions between the Aged Normal Group and the Interrupted Conditioning then Blast Group were obtained by subtracting the average DPOAE amplitudes of the group exposed to the traumatizing noise from the baseline DPOAE responses. Figure 5c provides a graphical representation of the mean amplitude differences between the two groups (in dB) plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The pattern of change of the DPOAE amplitude growth functions caused by the traumatizing noise exposure in animals previously sound conditioned on the interrupted schedule of moderate-level noise was similar to that obtained for the unconditioned animals (Figure 5a). Although similar, there were differences in the DPOAE responses between the unconditioned (Blast Only) group and the sound conditioned (Interrupted Conditioning then Blast) group. These differences are discussed in detail in Section 3.3.3.2 as they relate to the possible protective role of the interrupted sound conditioning exposure.

3.3.3 Comparison of the effects of the traumatizing noise

exposure on the DPOAE responses of unconditioned and sound conditioned animals -- Did sound conditioning provide protection?

When comparing the effects of the traumatizing noise exposure on the DPOAE amplitude growth function measured within the Blast Only Group, the Continuous Conditioning then Blast Group, and the Interrupted Conditioning then Blast Group, statistically significant differences were found between the exposure groups ($p=0.015$). Results show that overall (data pooled over frequency and intensity), the Interrupted Conditioning then Blast was least affected by the traumatizing noise, followed closely by the Blast Only Group and then the Continuous Conditioning then Blast Group. This implies that there was some amount of protection afforded by prior sound conditioning with the interrupted moderate-level noise exposure used in this study. However, the overall effect of the continuous sound conditioning protocol seemed to render the auditory system more susceptible to the traumatizing noise exposure.

Figure 6a provides a graphical representation of the mean amplitude differences (in dB) between the Blast Only Group and the Continuous Conditioning then Blast Group plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The positive excursions from zero represent an increased susceptibility to the traumatizing noise in the sound conditioned groups. This shows where (in terms of f_2 frequency and intensity) the sound conditioning acted to enhance the effects (DPOAE amplitude reductions) of the traumatic noise exposure rather than to protect against it. The negative excursions from zero show where the magnitudes of the DPOAE responses were actually larger in the sound conditioned groups than in the unconditioned control group. In other words, it is at these f_2 frequencies and corresponding primary tone levels that the sound conditioning appeared to provide protection against the subsequent traumatizing noise.

No exceptionally obvious pattern of protection was observed when comparing the DPOAE results of Blast Only Group and the Continuous Conditioning then Blast Group (Figure 6a). This means that there were no real definite frequency regions that demonstrated that the continuous conditioning noise was either helpful or harmful in protecting against the subsequent traumatic noise exposure. In addition, the amplitude differences between the two groups, for the most part, were fairly small, with maximum differences of 1-3 dB (on average). This finding is unusual given that the effects of continuous sound conditioning alone were more damaging to the DPOAE responses than the interrupted protocol.

Figure 6b provides a graphical representation of the mean amplitude differences (in dB) between the Blast Only Group and the Interrupted Conditioning then Blast Group plotted as a function of frequency for several different primary intensity levels (40-70 dB SPL). The positive excursions from zero represent an increased susceptibility to the traumatizing noise

in the sound conditioned groups. This shows where (in terms of f_2 frequency and intensity) the sound conditioning acted to enhance the effects (DPOAE amplitude reductions) of the traumatic noise exposure rather than to protect against it. The negative excursions from zero show where the magnitudes of the DPOAE responses were actually larger in the sound conditioned groups than in the unconditioned control group. In other words, it is at these f_2 frequencies and corresponding primary tone levels that the sound conditioning appeared to provide protection against the subsequent traumatizing noise.

An interesting finding was observed when comparing the DPOAE results of the Blast Only Group and the Interrupted Conditioning then Blast Group. Figure 10 illustrates that interrupted sound conditioning produced a dual effect on the DPOAE responses depending upon the f_2 test frequency. The results revealed an apparent trend towards an increased susceptibility to the traumatizing noise exposure in the sound conditioned animals in the lower test frequency range (f_2 = 707-2000 Hz) and some degree of protection in the test frequency range spanning f_2 frequencies of 2828-11312 Hz. The differences between the groups were statistically significant at f_2 frequencies of 707-, 1000-, 8000- and 11312 Hz ($P<0.05$). Still, although statistically significant differences were found in the two lowest and highest frequencies of f_2, it is the frequency-specific pattern showing where protection may or may not have occurred that is important and is worth further discussion.

4. Discussion

The purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous versus interrupted, moderate-level noise. Differences were determined by monitoring the changes that occurred in cubic ($2f_1$-f_2) distortion product otoacoustic emission (DPOAE) amplitude growth functions after a subsequent higher level traumatizing exposure in guinea pigs (*Cavia cobaya*) conditioned with either continuous or interrupted noise. DPOAEs were chosen for study because they are believed to reflect the mechanical properties of the cochlea, particularly as related to the status of outer hair cell (OHC) function (Mountain, 1980; Siegel and Kim, 1982; Siegel et al., 1982).

Focus was placed on answering two major experimental questions: (1) What was the effect of the sound conditioning protocols (continuous vs. interrupted) on DPOAE amplitude growth functions; and (2) Did prior sound conditioning provide protection against the damaging effects of the traumatizing noise exposure, and if so, was one sound conditioning protocol more effective?

4.1 Effects of sound conditioning on DPOAE responses

4.1.1 Continuous sound conditioning

The continuous sound conditioning exposure protocol used in this study caused reductions in DPOAE amplitudes in a frequency-

and intensity-dependent manner (Figure 3a). The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and higher frequencies. Specifically, the greatest amplitude reductions (approximately 12-18 dB) occurred in the frequency range spanning f_2 frequencies of 1414-4000 Hz. This frequency region corresponds to the center and upper cutoff frequency of the noise exposure band used in this study (octave band noise; 1.0-2.0 kHz), and extended to one octave above the upper cutoff frequency. This finding is not surprising, because it is consistent with previous reports showing that the maximum effect of an exposure can occur in the same frequency region of the exposure and/or approximately ½-1 octave above the upper cutoff frequency for bands of noise of reasonably constant spectrum level (Davis et al., 1943; Ward, 1976; Mitchell et al., 1977; Salvi et al., 1982). In addition, in most cases, the greatest decreases in amplitude occurred for primary levels ranging from 40-60 dB SPL. This result is consistent with the idea that DPOAEs elicited by low-to-moderate level primary tones are physiologically vulnerable to the oto-traumatic effects of the noise exposure (Kim, 1980; Zurek et al., 1982; Subramaniam et al., 1994a,b). It is within this intensity range that the active, nonlinear processes of the OHCs normally exert their greatest influence. Puel et al. (1988a) speculated that it is the active process that is affected first during acoustic trauma.

The frequency-specific changes in the DPOAE response measurements caused by the continuous sound conditioning protocol used in this study are supported by other evidence in the literature (Ryan et al., 1994; Fowler et al., 1995). In the studies performed by Ryan et al. (1994) and Fowler et al. (1995), auditory brainstem response thresholds were measured after the final day of a long-term, moderate-level noise exposure. The results of both experiments demonstrated a pattern of noise-induced threshold shift similar to the pattern of DPOAE amplitude growth function alterations measured in the present study (Figure 3a). That is, the maximal effect of the continuous noise exposure occurred at test frequencies within and slightly above the noise exposure band, with lesser effects outside of this frequency region.

The results of the present study demonstrating changes in DPOAE amplitude growth functions in guinea pigs after exposure to long-term continuous moderate-level noise were different from the findings of Canlon and Fransson (1995), who also tested the effects of continuous sound conditioning on DPOAEs in guinea pigs. These authors reported that their continuous sound conditioning protocol (81 dB SPL, 1 kHz pure-tone presented continuously for 24 days) did not cause any significant alterations in $2f_1$-f_2 DPOAE amplitudes at frequencies of 1.75-, 2.1-, 2.8-, and 3.5 kHz. A possible explanation for the discrepancy between the results of the two studies might be that the actual DPOAE frequencies chosen for study by Canlon and Fransson (1995) were not generated within the region along the

cochlear partition that was affected by the 1 kHz pure tone conditioning exposure. Again, the region of DPOAE generation is thought to be near, or at, the f_2 place on the cochlear partition (Matthews and Molnar, 1986; Brown et al., 1992; Allen and Fahey, 1993; Puel et al., 1995). The lowest frequency DPOAE tested by Canlon and Fransson was $2f_1-f_2$ = 1.75 kHz (f_2/f_1=1.225; f_1=2.258 kHz, f_2=2.766 kHz). The frequency of f_2 in this case is approximately 1½ octaves above the frequency of the pure-tone, moderate-level conditioning exposure. This means that if the exposure was such that it caused only very localized changes in cochlear function in the region of the 1 kHz conditioning tone, then it would be expected that DPOAEs with primary frequencies (especially f_2) outside of this region would be, for the most part, unaltered by the exposure. Recently, Skellett et al. (1996) showed frequency-dependent and very localized reductions in the $2f_1-f_2$ DPOAE amplitude growth functions of guinea pigs exposed continuously for 3 and 11 days to a noise with spectral characteristics similar to the one used in this study (1.1 - 2.0 kHz). The intensity level of this noise exposure, however, was only 65 dB SPL. These reductions occurred only when the frequency of f_2 was within the noise exposure band.

4.1.2 Interrupted sound conditioning

The interrupted conditioning exposure also caused reductions in DPOAE amplitudes in a frequency- and intensity-dependent manner (Figure 3b). The maximum effect of the noise exposure occurred in the mid-frequency range of the frequencies tested, while effects were not as great in the lower and virtually non-existent in the higher frequencies. For this exposure protocol however, the greatest amplitude reductions occurred in a narrower frequency range (relative to the continuous sound conditioning protocol), spanning f_2 frequencies of 2000-4000 Hz. The magnitude of the reductions within this range was approximately 11-16 dB. This frequency region corresponds to the upper cutoff frequency of the noise exposure band used in this study (octave band noise; 1.0-2.0 kHz) and extended to one octave above the upper cutoff frequency. Within this frequency region, the largest decreases in DPOAE amplitude occurred for primary levels ranging from about 40-60 dB SPL. This result is consistent with the idea that DPOAEs elicited by low-to-moderate level primary tones are physiologically vulnerable to the oto-traumatic effects of the noise exposure (Kim, 1980; Zurek et al., 1982; Subramaniam et al., 1994a,b). It is within this intensity range that the active, nonlinear processes of the OHCs normally exert their greatest influence.

At the lower f_2 frequencies (707- and 1000 Hz), there were actually intensity levels of the amplitude growth functions where the DPOAEs amplitudes recorded for the sound conditioned group were larger than those of the normal control group (Figure 2a and 2b). These f_2 frequencies coincide with the lower cutoff frequency of the noise exposure band (1000 Hz) and a frequency ½ octave below it (707 Hz). These elevations in the DPOAE

amplitudes relative to the normal controls occurred for primary levels ranging from 50-60 dB SPL. Elevated DPOAE amplitudes measured in sound conditioned animals at frequencies bordering the noise exposure band have also been reported by Boettcher and Schmiedt (1995). Similarly, the amplitude elevations occurred for primary levels of 50 and 60 dB SPL. When studying the effects of noise-induced hearing loss on DPOAEs in humans, Kim et al. (1992) found that DPOAE amplitudes were larger than normal at the edge of a hearing loss in subjects who exhibited audiograms that were typical of those obtained in subjects with prior histories of noise exposure (4 kHz "notch"). While not conclusive, the results of this and the other two studies mentioned suggest that the elevated DPOAE amplitudes measured in the frequency region bordering a noise-induced cochlear lesion may be related to the effects of the noise exposure on the mechanism(s) responsible for DPOAE generation.

Other researchers have also studied the effects of interrupted sound conditioning protocols on DPOAEs. Subramaniam et al. (1994a,b; 1995) examined the changes in DPOAE amplitude growth functions of chinchillas in response to both low- and high-frequency interrupted sound conditioning protocols. When the high-frequency interrupted noise (octave band noise centered at 4.0 kHz) was used by Subramaniam et al. (1995), the results were similar to the changes observed in the DPOAE amplitude growth functions of the present study in that by the final day of the interrupted exposure, the reductions in the DPOAE amplitudes were fairly localized to frequencies within the noise exposure band.

However, the pattern of the DPOAE amplitude reductions demonstrated in the present experiment was different from the results of both studies performed by Subramaniam et al. (1994 a,b) when they used a low-frequency conditioning exposure. The results of the present study showed a relatively predictable pattern of DPOAE loss in that the maximum loss was confined to the frequency region very near the frequency band of the noise exposure. Again, this region corresponded to the upper cutoff frequency of the noise exposure band used in this study (octave band noise; 1.0-2.0 kHz) and extended to one octave above the upper cutoff frequency. Small reductions in DPOAE amplitudes were found outside of this region, especially in the higher frequencies tested. Although the noise used by Subramaniam et al. was a low frequency noise (octave band noise centered at 0.5 kHz), there were substantial reductions in the DPOAE amplitudes even at high frequencies, i.e. 4 and 8 kHz. This spread of cochlear damage in response to low frequency noise exposure has been previously observed (Zurek et al., 1982; Clark et al.,1987; Subramaniam et al., 1991a).

4.1.3 Comparison of the effects of the continuous and interrupted sound conditioning protocols -- Testing the validity of the EEH

Although the two sound conditioning protocols used in the

present experiment had equal acoustic energy, the resultant changes in DPOAE amplitude growth functions measured in the groups of guinea pigs exposed to either continuous or interrupted noise were not equivalent. Figure 3a and 3b show the mean DPOAE amplitude differences between the Continuous Conditioning Group and the Aged Normal Group, and the Interrupted Conditioning Group and the Aged Normal Group, respectively. When comparing these figures, it becomes apparent that overall, the interrupted conditioning noise was significantly less damaging to the cochlea than the continuous conditioning noise. Not only were the magnitudes of the noise-induced DPOAE amplitude reductions less in the animals conditioned with the interrupted noise as opposed to the continuous noise, but the range of f_2 frequencies affected by the interrupted exposure was also decreased. Therefore, these results do not support the validity of the EEH which assumes that the cumulative damage to the auditory system is a function of the <u>total</u> acoustic energy received, regardless of the distribution of energy over time (Eldred et al., 1955). This finding is backed by previous histological data showing that while the pattern of hair cell damage was the same for a continuous vs. an interrupted noise exposure (both with equal acoustic energy), the magnitude of hair cell damage induced by the exposures was less for the interrupted exposure (Bohne et al., 1985, 1987; Fredelius and Wersäll, 1992). More recently, Chang and Norton (1996) reported that an intermittent noise exposure caused less reductions in DPOAE amplitudes than a continuous noise exposure, a finding similar to that found in the present study. Thus, it is quite possible that the rest (quiet) periods within the interrupted exposure acted to minimize noise-induced hair cell damage and were responsible for the significantly smaller reductions in the DPOAE amplitudes (when compared to the continuous exposure) observed in this study.

4.2 Did prior sound conditioning provide protection against the damaging effects of the traumatizing noise exposure, and if so, was one sound conditioning protocol more effective?

4.2.1 Continuous sound conditioning

There was no exceptionally obvious pattern of protection provided by the continuous conditioning exposure (Figure 6a). Thus, the overall effect of the continuous sound conditioning protocol seemed to render the auditory system more susceptible to the traumatizing noise exposure. The lack of protection demonstrated here could possibly be related to the fact that the moderate-level continuous conditioning exposure chosen for study may have, by itself, been enough to cause irreversible damage to the outer hair cells, thus precluding the possibility of providing protection against the damaging effects of the subsequent traumatizing exposure. It was not determined whether or not this conditioning exposure had any residual effects on the DPOAE amplitude growth functions after the one week rest period that was given. However, it has been suggested that protection against subsequent noise trauma may be less likely to occur in

the presence of a threshold shift induced by the sound conditioning exposure (Canlon et al, 1992; Ryan et al., 1994; Canlon and Fransson, 1995). The results of other continuous sound conditioning experiments showed that when the moderate-level conditioning noise did not cause any morphological or physiological alterations to the cochlea, no rest period was required before the subsequent traumatizing noise in order for a protective effect to exist (Canlon et al., 1988,1992; Canlon and Fransson, 1995).

Thus, it is possible that the continuous sound conditioning exposure used in this study (1-2 kHz; 89 dB SPL; presented continuously for 11 days) caused some degree of permanent damage to the region of the cochlea spanning the f_2 frequency range of interest. This may have resulted in the lack of any consistent and sizable protective effect due to prior sound conditioning with a continuous moderate-level noise.

4.2.2 Interrupted sound conditioning

There was some degree of protection afforded by prior sound conditioning with the interrupted, moderate-level noise exposure. Sound conditioning with the interrupted protocol prior to exposure to the traumatic noise exposure produced a dual effect on the DPOAE responses depending upon the f_2 test frequency (Figure 6b). The results revealed an apparent trend towards an increased susceptibility to the traumatizing noise exposure in the sound conditioned animals in the lower test frequency range (f_2 = 707-2000 Hz) and some degree of protection in the test frequency range spanning f_2 frequencies of 2828-11312 Hz. This lack of protection in the frequency range encompassing the noise exposure band and the existence of protection in the frequency range above the noise exposure band in guinea pigs conditioned with an interrupted moderate-level noise has not been previously reported. The frequency-related pattern of protection which has been reported by other investigators usually shows that there <u>is</u> significant protection from the subsequent traumatizing exposure afforded by prior sound conditioning with an interrupted protocol within the frequency region of the exposure band which further extends to higher frequencies (Campo et al., 1991; Henderson et al., 1992; Subramaniam et al., 1992, 1993). As mentioned earlier, the lack of protection demonstrated in this study for the lower frequency region (coinciding with the noise exposure band) could possibly be related to the fact that the moderate-level interrupted conditioning noise used may have, by itself, caused irreversible damage to the outer hair cells within this frequency region. Permanent damage to the cells within this region of the cochlea may have precluded the protective role of the sound conditioning exposure, and rendered the auditory system more susceptible to the deleterious effects of subsequent noise trauma. Again, as for the continuous conditioning protocol, it was not determined whether or not this interrupted conditioning exposure had any residual effects on the DPOAE amplitude growth functions after the one week rest period that was interposed

between the conditioning and the traumatic exposures.

4.2.3 Comparison of the effects of the traumatizing noise exposure on the DPOAE responses of animals conditioned with continuous vs. interrupted moderate-level noise -- Is either sound conditioning protocol more protective?

The primary purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous versus interrupted moderate-level noise. When comparing the effects of the traumatizing noise exposure on the DPOAE responses of the sound conditioned groups of animals, it was found that the group least affected by the high-level noise depended upon the frequency of f_2 being tested. Within the low frequency test region (f_2=707, 1000, 1414, and 2000 Hz), the results suggest that the traumatizing noise produced slightly more damage in the animals previously conditioned with the interrupted schedule of moderate-level noise. At higher frequencies of f_2 (2828-11,312 Hz), however, smaller amplitude reductions were found in the DPOAE responses of the Interrupted Conditioning then Blast Group as compared with the reductions measured in the Continuous Conditioning then Blast Group. Thus, the damaging effects of the traumatizing noise were smaller in the animals previously conditioned with the interrupted schedule of moderate-level noise within this higher frequency range.

The results of the present study suggest that <u>overall</u>, there were significant differences in the degree of protection provided by prior sound conditioning with continuous vs. interrupted schedules of moderate-level noise of equal acoustic energy. Given the noise exposure protocols chosen for study, it appears that the interrupted sound conditioning protocol provided more protection against the damaging effects of the subsequent traumatic exposure than the continuous conditioning protocol. While no real definite frequency regions were identified demonstrating that continuous sound conditioning was either helpful or harmful in protecting against the subsequent traumatic noise exposure (Figure 6a), the effectiveness of the interrupted sound conditioning protocol in providing protection was highly frequency-dependent (Figure 6b). In the frequency range encompassing the noise exposure band and extending down to ½ octave below the lower cutoff frequency (f_2 = 707-2000 Hz), neither sound conditioning exposure was effective in providing protection against the subsequent traumatizing exposure when the DPOAE responses were compared with those obtained in the unconditioned group. However, when the frequencies of f_2 generating the DPOAEs were limited to the frequency region above that of the noise exposure band, the results indicate that some degree of protection was afforded by the interrupted sound conditioning protocol. Although there have been other attempts to compare the effectiveness of the two different schedules of moderate-level conditioning noise (Fowler et al., 1995; White et al., 1996), neither of these investigations produced results

which demonstrated that either of the conditioning protocols used provided any degree of protection against the subsequent traumatic exposure.

5. Summary and Conclusions

The primary purpose of this study was to test the hypothesis that differences exist in the amount of protection provided by prior sound conditioning with continuous versus interrupted schedules of moderate-level noise of equal acoustic energy. Differences were determined by monitoring the changes that occurred in distortion product otoacoustic emission (DPOAE) amplitude growth functions after a subsequent higher level traumatizing exposure in guinea pigs (*Cavia cobaya*) conditioned with either continuous or interrupted noise. The results suggest that overall, there were significant differences in the degree of protection provided by prior sound conditioning with the continuous and interrupted schedules of moderate-level noise used in this study. Specifically, there was some degree of protection afforded by prior sound conditioning with the interrupted noise protocol. The frequency region where protection was found, however, was limited to the region above that of the noise exposure band. Conversely, there was a lack of any consistent and sizable protective effect found across the entire test frequency range when the continuous conditioning protocol was used as the moderate-level exposure. Therefore, while neither conditioning protocol was effective in providing protection against damaging effects of the subsequent traumatizing exposure in the low frequency range (frequencies encompassing the noise exposure band and extending down to ½ octave below the lower cutoff frequency), the interrupted sound conditioning protocol was more effective than the continuous conditioning protocol in the frequency region above that of the noise exposure band.

One possible reason for the lack of protection found in this study in response to prior sound conditioning (across most test frequencies for the continuous conditioning protocol and in the frequency region encompassing the noise exposure band for the interrupted conditioning protocol) is that the moderate-level conditioning exposures may have, by themselves, caused irreversible damage to the outer hair cells within the test frequency region. Permanent damage to the outer hair cells within this region of the cochlea may have precluded the protective role of the sound conditioning exposures, and rendered the auditory system more susceptible to the deleterious effects of the subsequent noise trauma. This explanation is supported by the findings of Canlon et al. (1992) which suggested that protection against subsequent noise trauma was less likely to occur in the presence of a threshold shift induced by the sound conditioning exposure. It was not determined whether or not the sound conditioning protocols used in the present study had any residual effects on the DPOAE responses after the one week rest period that was given in between the conditioning and the traumatic exposures. Future experiments will include the

addition of exposure groups whose DPOAE responses will be measured after the one week rest period so that information can be obtained about the condition of the outer hair cells just prior to the traumatic noise exposure.

Many investigators have studied the protective effect of different schedules of moderate-level noise on the auditory system in a variety of animal species (guinea pigs, Canlon et al., 1988, 1992; Canlon and Fransson, 1995; rabbits, Canlon et al., 1992; chinchillas, Campo et al., 1991; Henderson et al., 1992 Subramaniam et al., 1992, 1993a,b; gerbils, Ryan et al., 1994; mice, Fowler et al., 1995). Both continuous and interrupted schedules of moderate-level noise have been shown to be effective in providing protection against the damaging effects of subsequent noise trauma. However, there have also been reports suggesting that similar schedules (continuous and interrupted) of moderate-level noise exposure, in some cases, render the auditory system more susceptible to the ototoxic effects of the higher-level subsequent exposure (ref.). The results of the present study <u>again</u> support the protective role of an interrupted sound conditioning exposure protocol. However, unlike the findings of other studies using similar conditioning protocols (Campo et al., 1991; Henderson et al., 1992; Subramaniam et al., 1993a), the effectiveness of the interrupted moderate-level noise exposure in providing protection was highly frequency-dependent, limited only to the frequency region above that of the noise exposure band. In addition, while other investigators have demonstrated the protective role of continuous sound conditioning (Canlon et al., 1988, 1992; Ryan et al., 1994; Canlon and Fransson, 1995), the results of this study do not support their findings.

Given the disparate findings of this and other studies, it appears that the protective effect of prior sound conditioning with moderate-level noise is not a very straightforward phenomenon and is highly dependent on the noise exposure conditions (e.g. frequency, duration, intensity), animal species, and response measurements (e.g. behavioral thresholds, auditory brainstem response thresholds, DPOAE amplitudes) studied. Additional studies are needed so that a better understanding of the ideal exposure conditions for achieving noise-induced resistance to hearing loss can be obtained.

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Table 1. Primary tones and corresponding DPOAEs (measured in Hz)

f_1	f_2	$2f_1-f_2$
589	707	471
833	1000	667
1178	1414	943
1667	2000	1333
2357	2828	1886
3333	4000	2667
4713	5656	3770
6667	8000	5334
9427	11312	7542

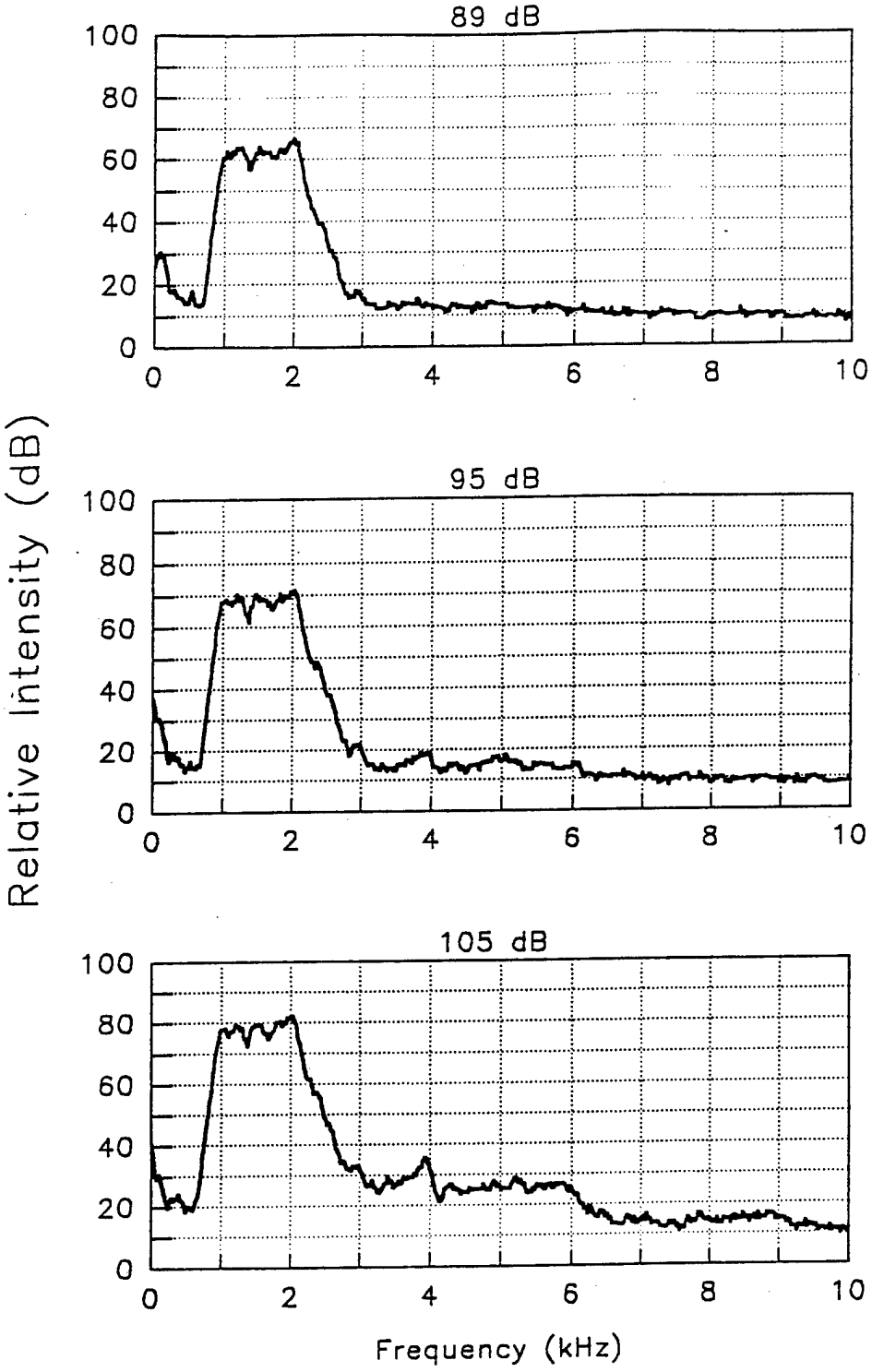

Figure 1 Spectral characteristics of the continuous conditioning noise (89 dB SPL; top), the interrupted conditioning noise (95 dB SPL; middle), and the traumatizing noise (105 dB SPL; bottom) exposures. The graphs represent the relative intensity of the noise exposures (dB) plotted as a function of frequency (kHz).

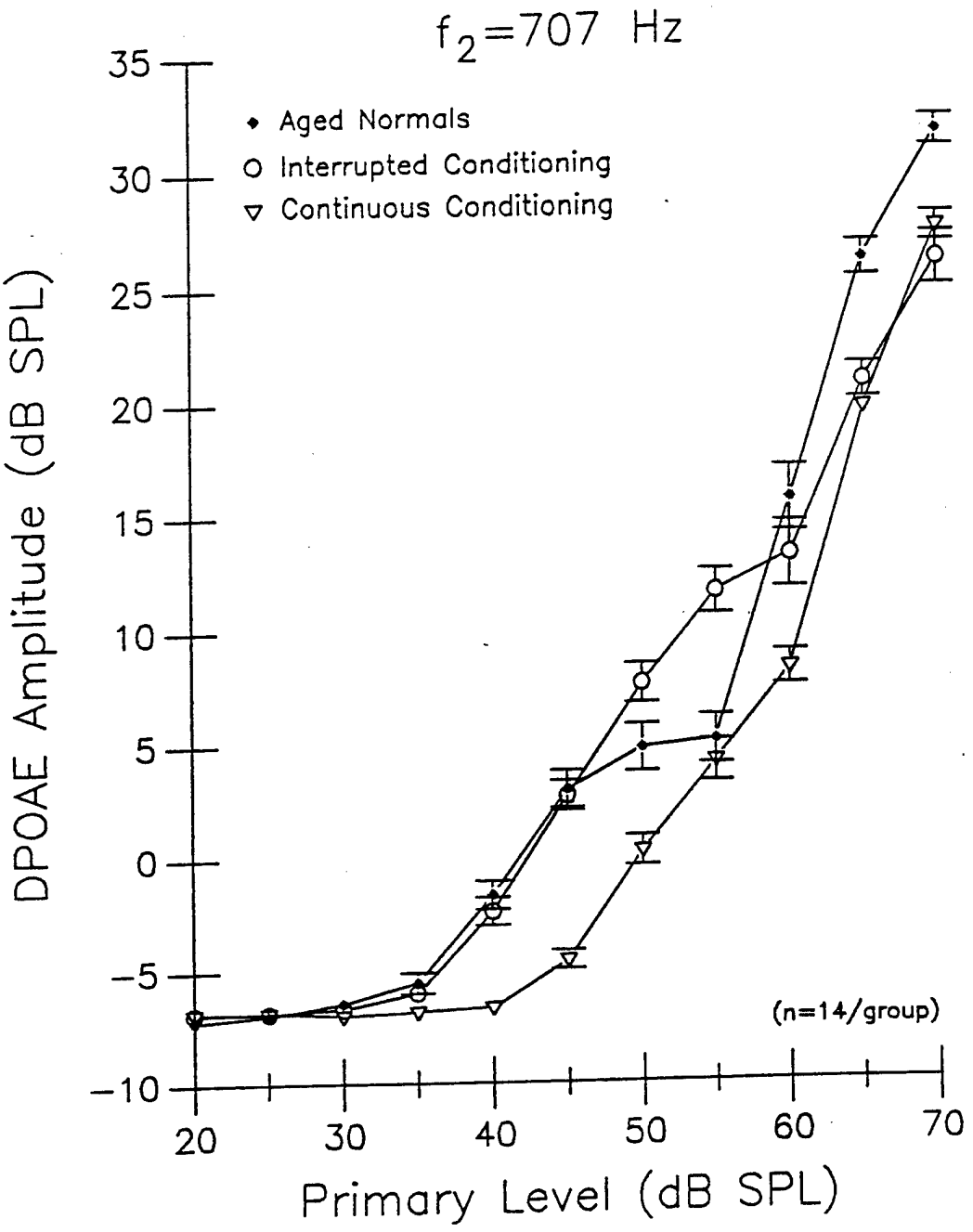

Figure 2a. The effect of sound conditioning on DPOAE responses at f_2 = 707 Hz. DPOAE amplitude growth functions obtained from the Continuous Conditioning Group (n=14; open down-triangles) and the Interrupted Conditioning Group (n=14; open circles) are plotted along with the functions of the Aged Normal Group (n=14; closed circles). Data are presented as mean DPOAE amplitude ± S.E. as a function of primary level (20-70 dB SPL).

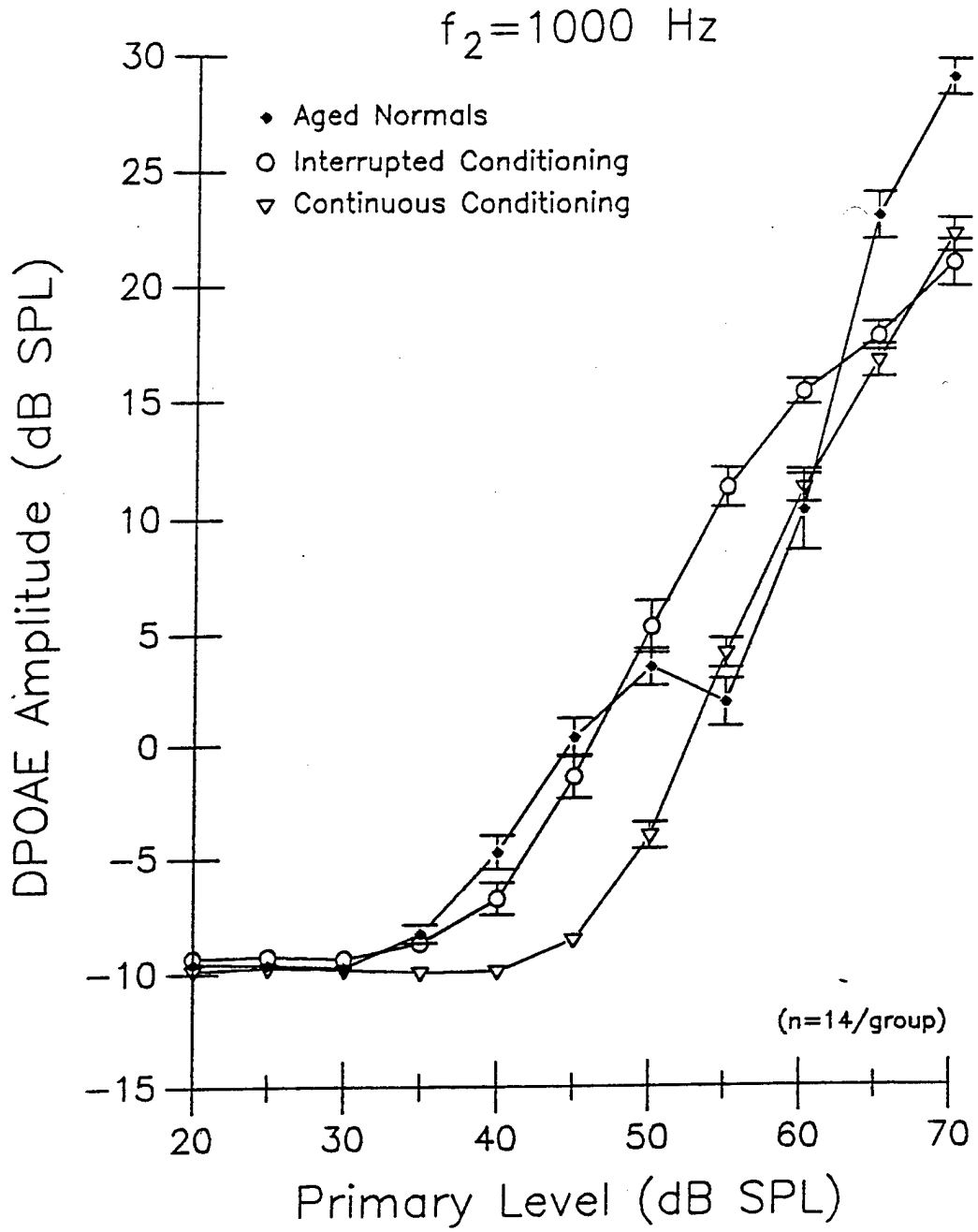

Figure 2b. The effect of sound conditioning on DPOAE responses at f_2 = 1000 Hz. For additional information, see legend for Figure 2a.

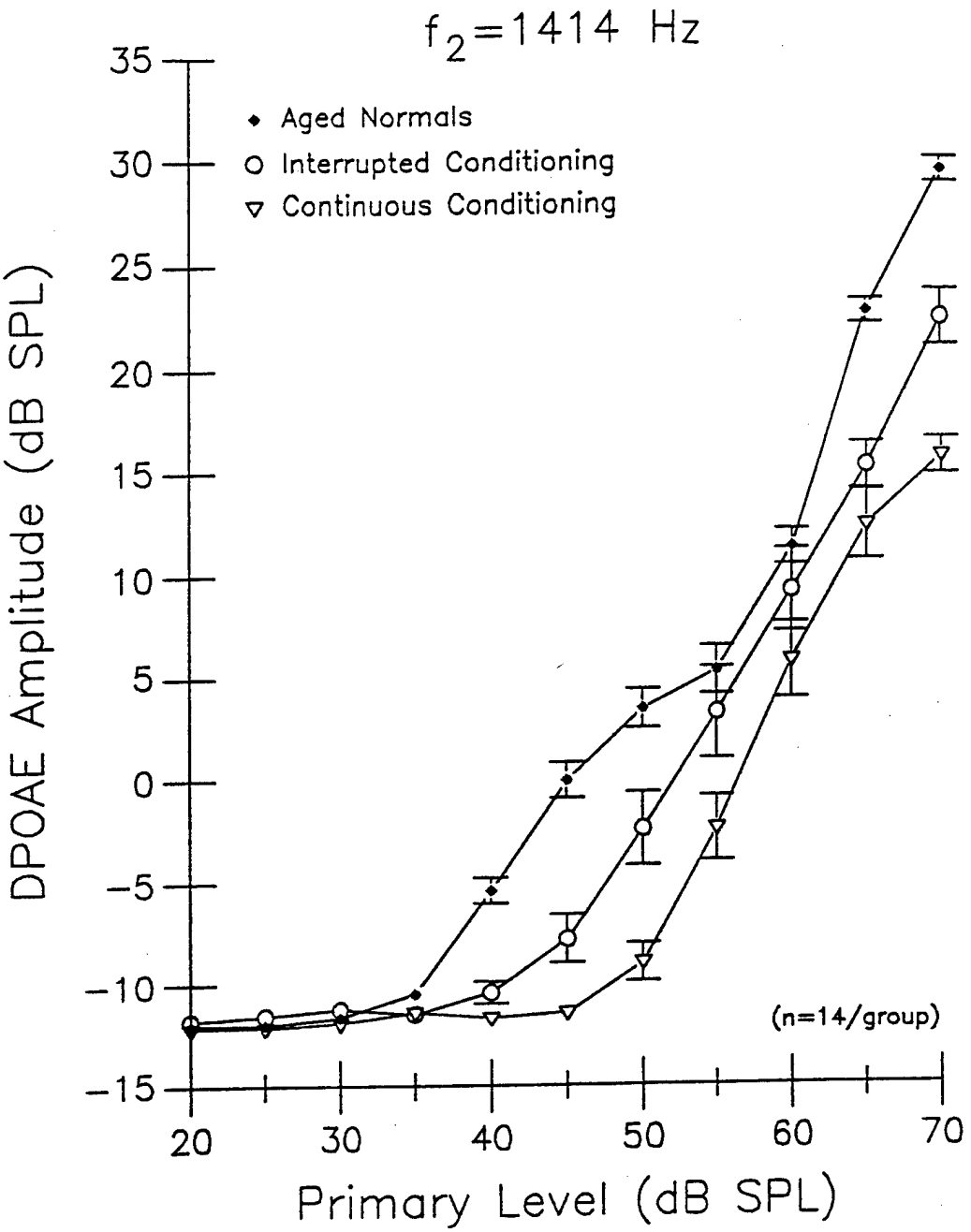

Figure 2c. The effect of sound conditioning on DPOAE responses at f_2 = 1414 Hz. For additional information, see legend for Figure 2a.

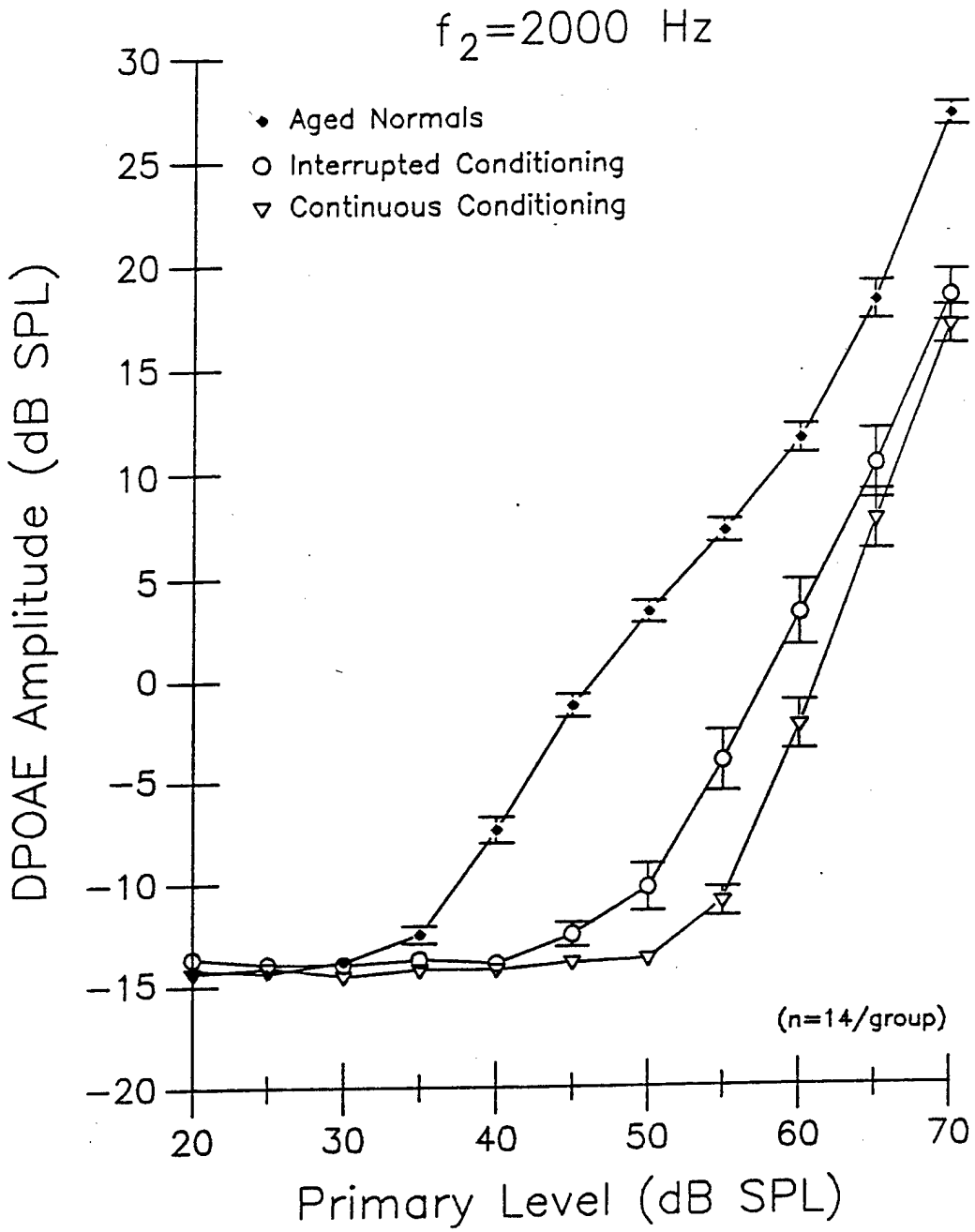

Figure 2d. The effect of sound conditioning on DPOAE responses at f_2 = 2000 Hz. For additional information, see legend for Figure 2a.

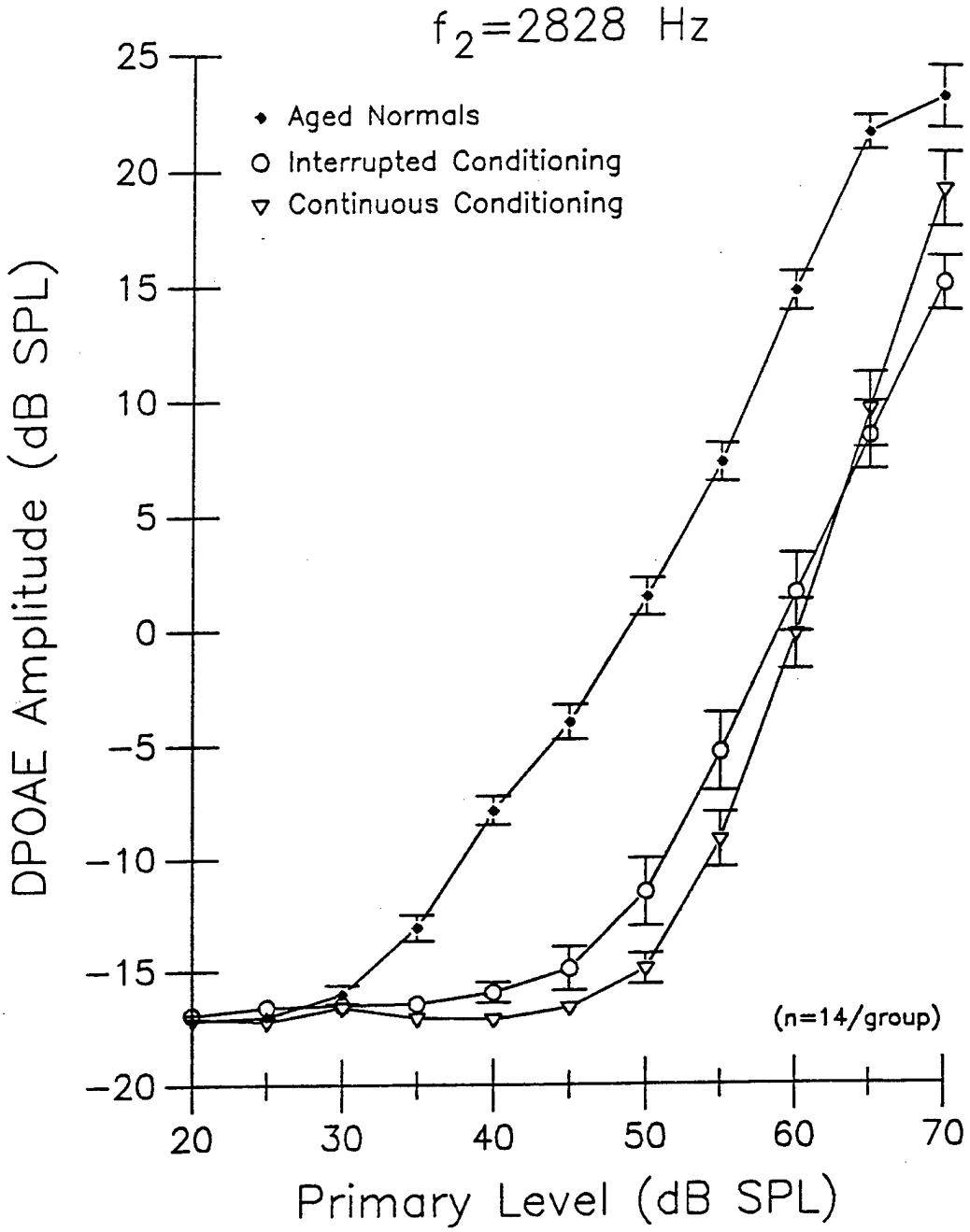

Figure 2e. The effect of sound conditioning on DPOAE responses at f_2 = 2828 Hz. For additional information, see legend for Figure 2a.

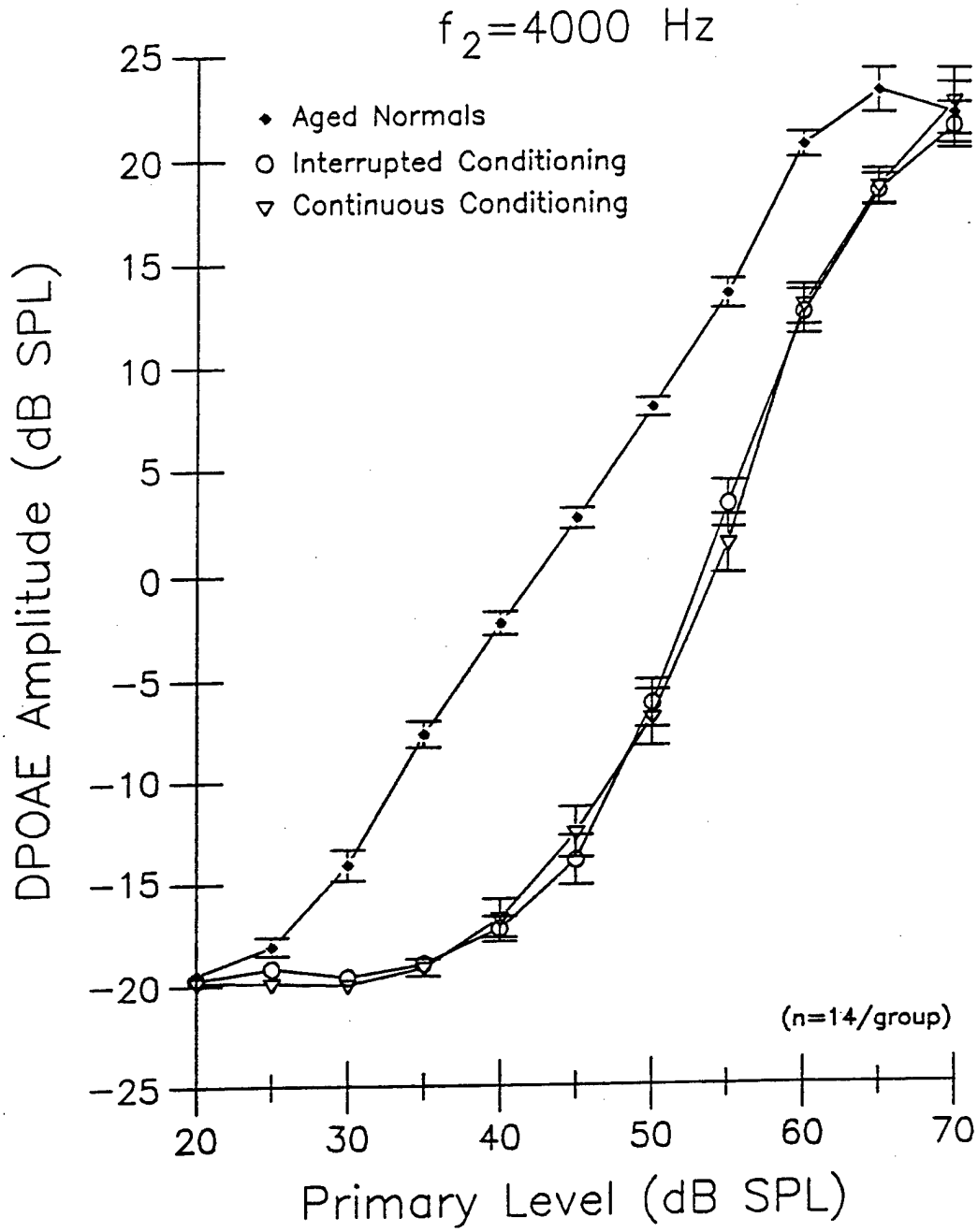

Figure 2f. The effect of sound conditioning on DPOAE responses at f_2 = 4000 Hz. For additional information, see legend for Figure 2a.

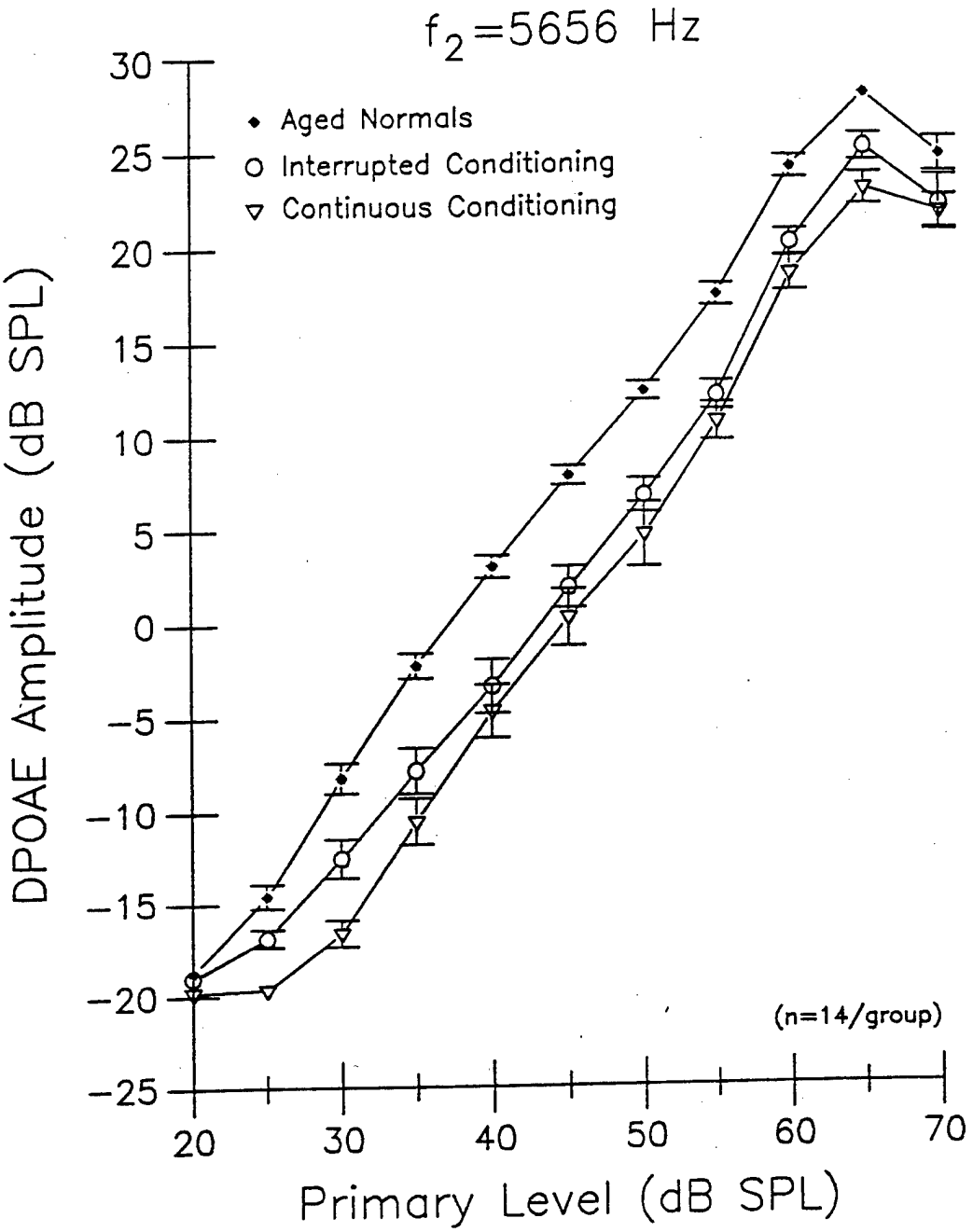

Figure 2g. The effect of sound conditioning on DPOAE responses at f_2 = 5656 Hz. For additional information, see legend for Figure 2a.

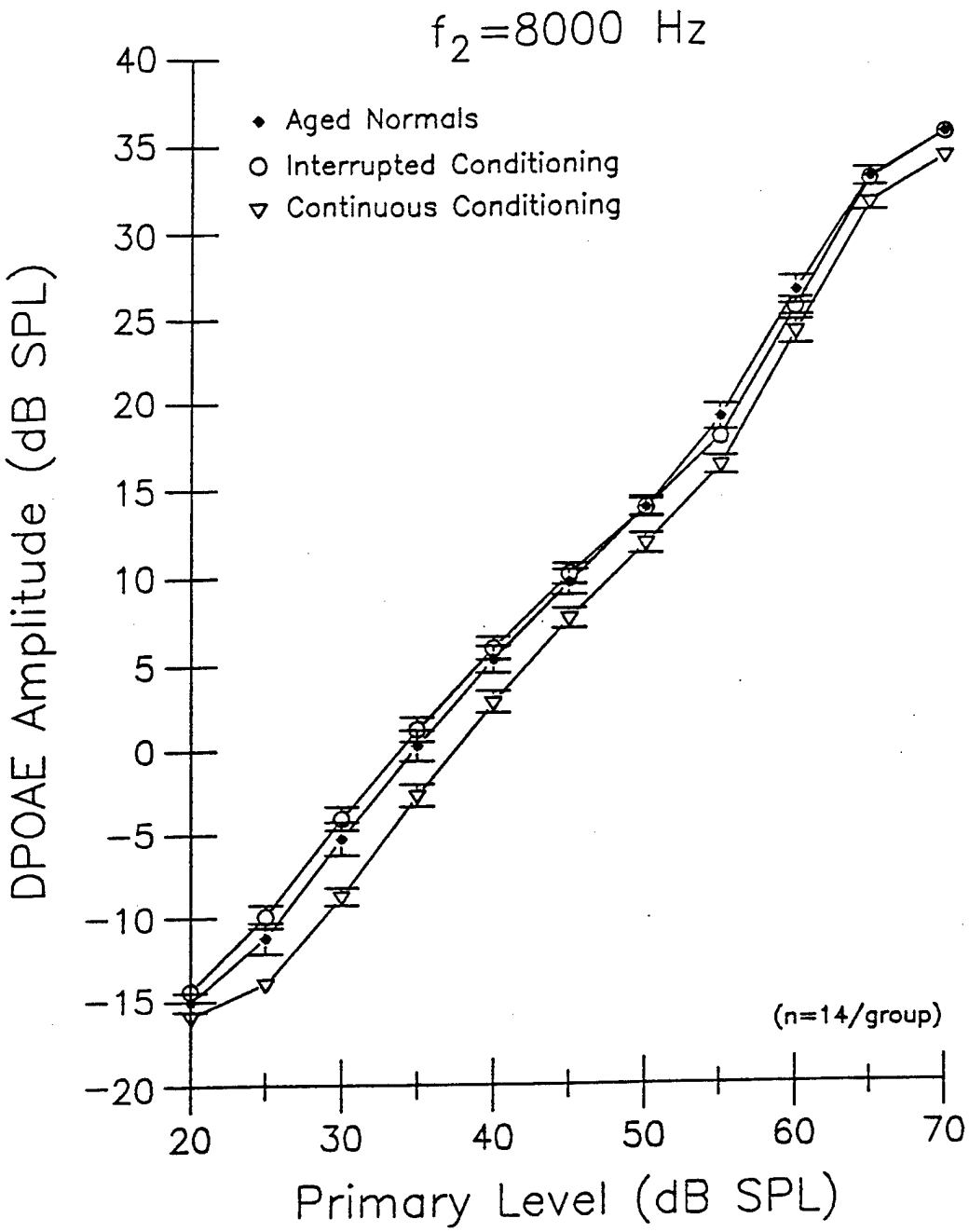

Figure 2h. The effect of sound conditioning on DPOAE responses at f_2 = 8000 Hz. For additional information, see legend for Figure 2a.

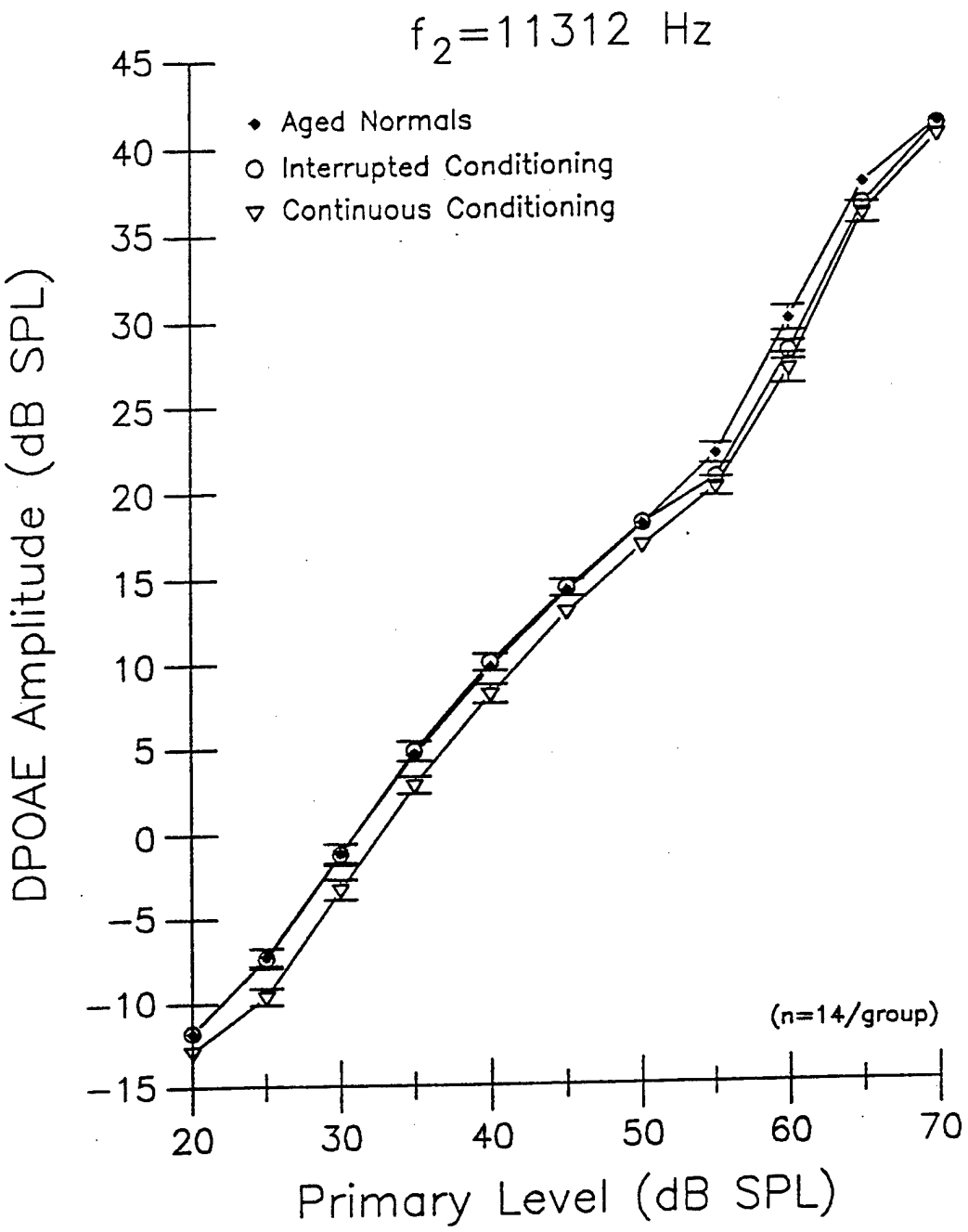

Figure 2i. The effect of sound conditioning on DPOAE responses at f_2 = 11312 Hz. For additional information, see legend for Figure 2a.

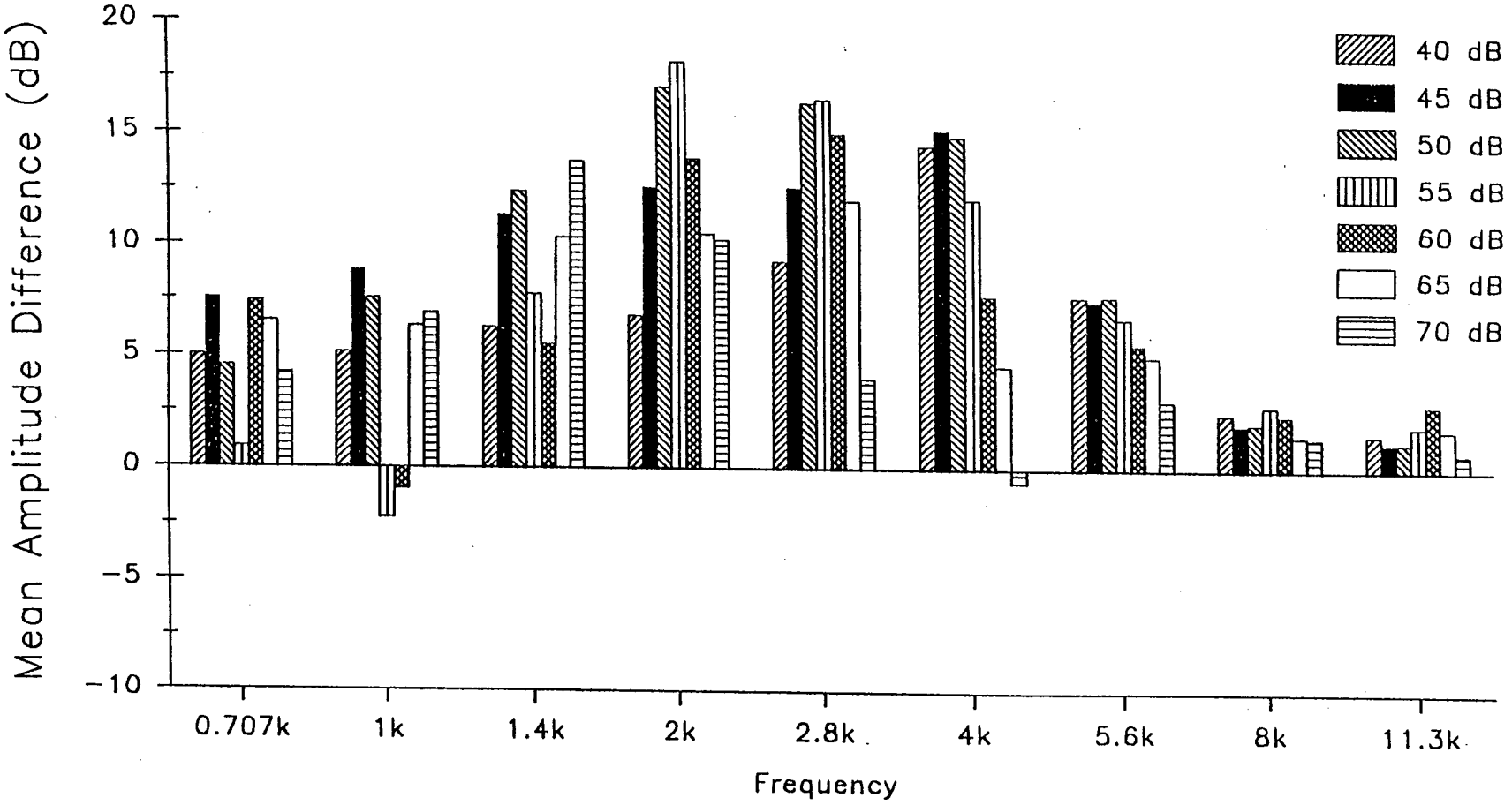

Figure 3a The effect of continuous sound conditioning on normal DPOAE responses. Data are presented as the mean amplitude differences between the Aged Normal Group and the Continuous Conditioning Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

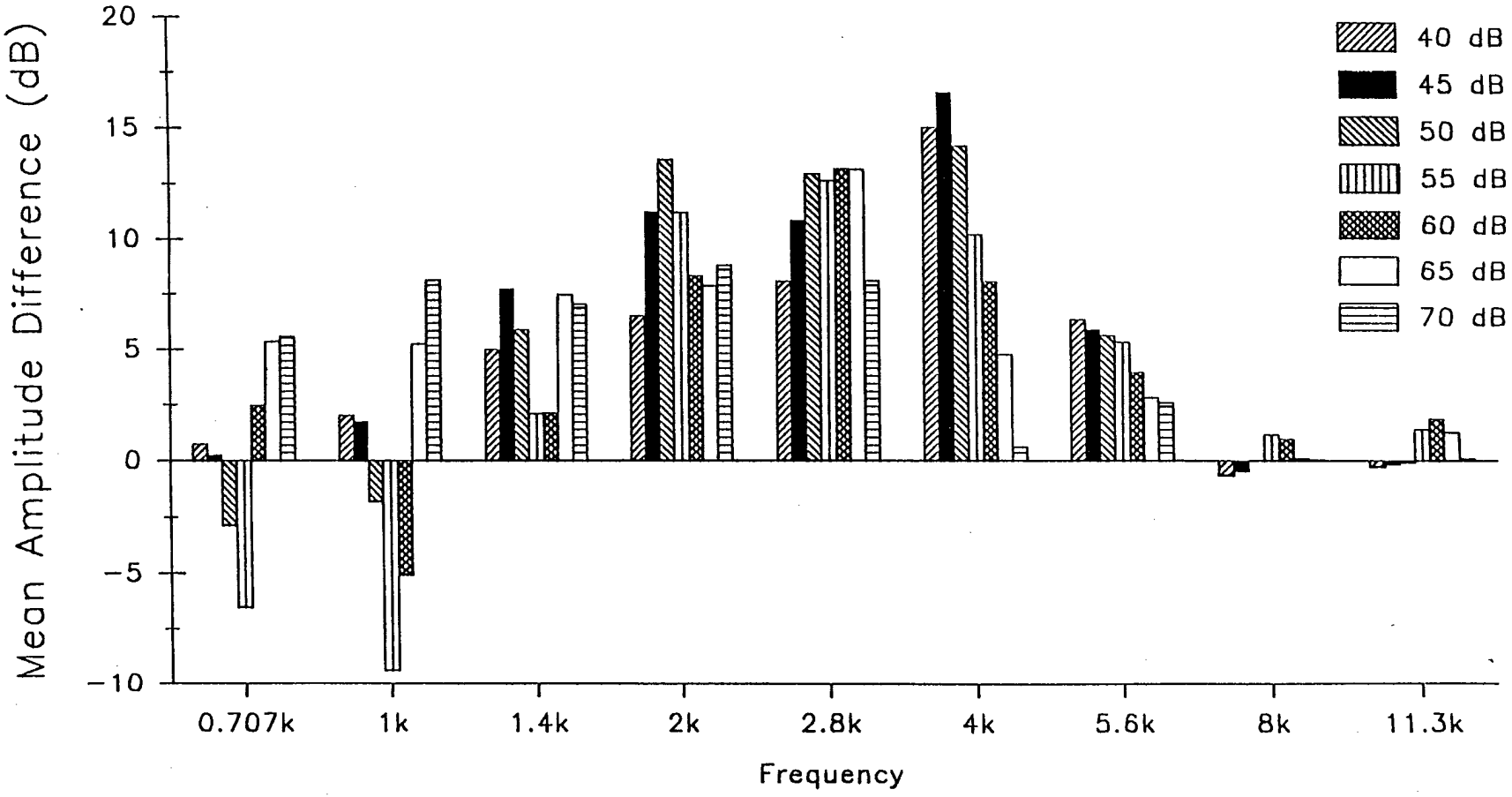

Figure 36 The effect of interrupted sound conditioning on normal DPOAE responses. Data are presented as the mean amplitude differences between the Aged Normal Group and the Interrupted Conditioning Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

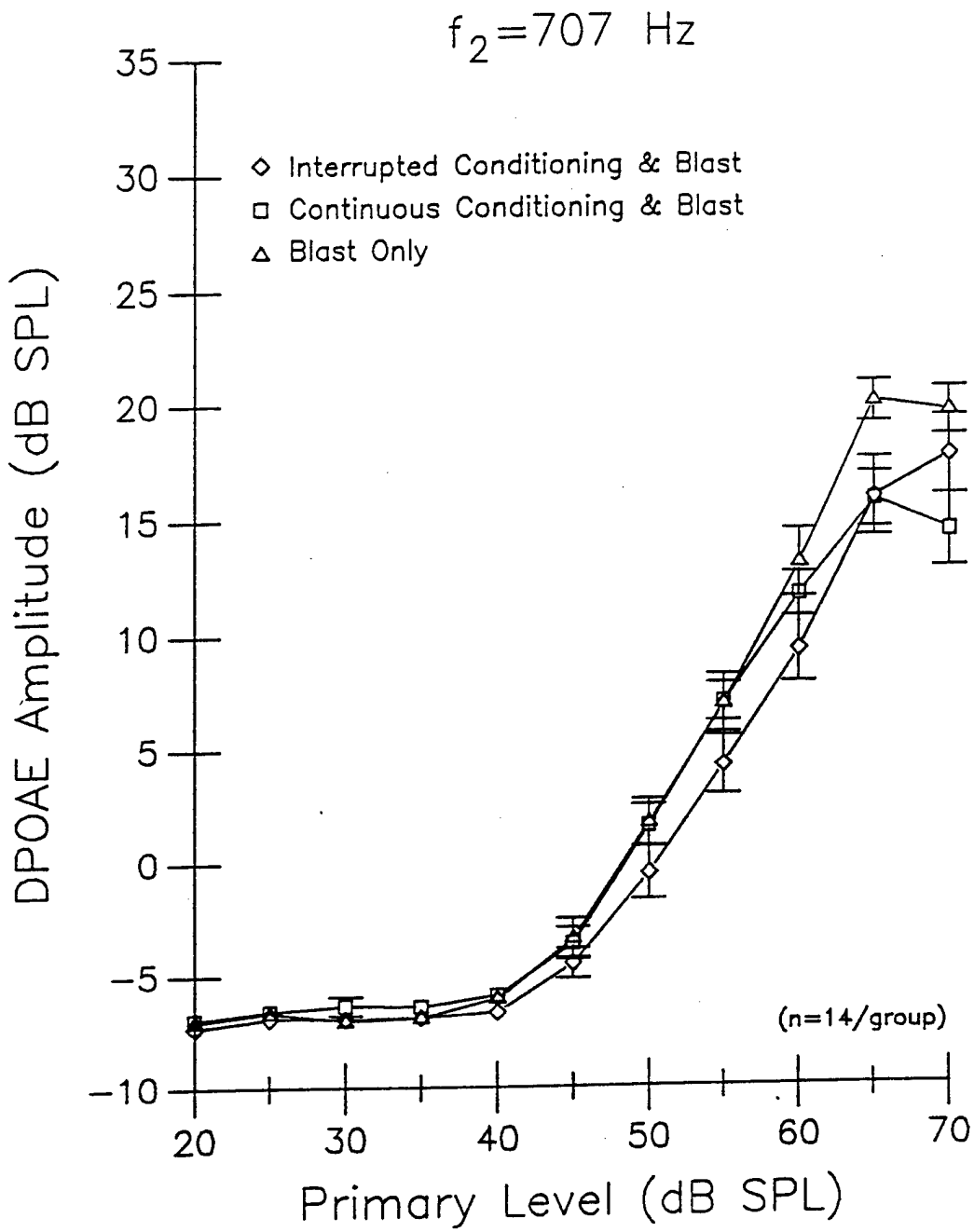

Figure 4a The effect of the traumatizing noise exposure on the DPOAE responses of unconditioned and sound conditioned animals at f_2 = 707 Hz. The individual plots represent the DPOAE amplitude growth functions obtained from the Blast Only Group (n=14; open up-triangles), the Continuous Conditioning then Blast Group (n=14; open squares), and the Interrupted Conditioning then Blast Group (n=14; open diamonds). Data are presented as mean DPOAE amplitude ± S.E. as a function of primary level (20-70 dB SPL).

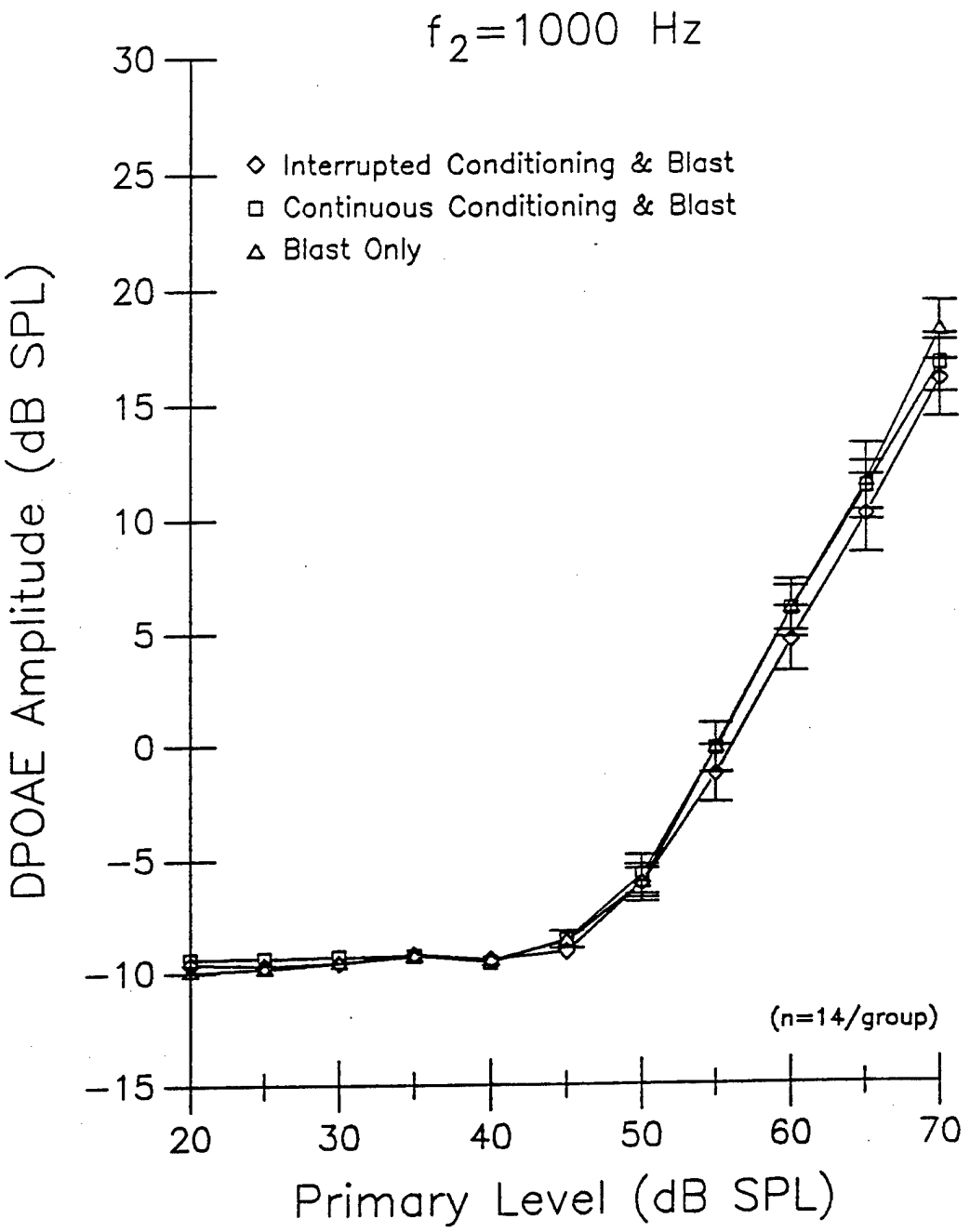

Figure 4b The effect of the traumatizing noise exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 1000 Hz. For additional information, see legend for Figure 3a.

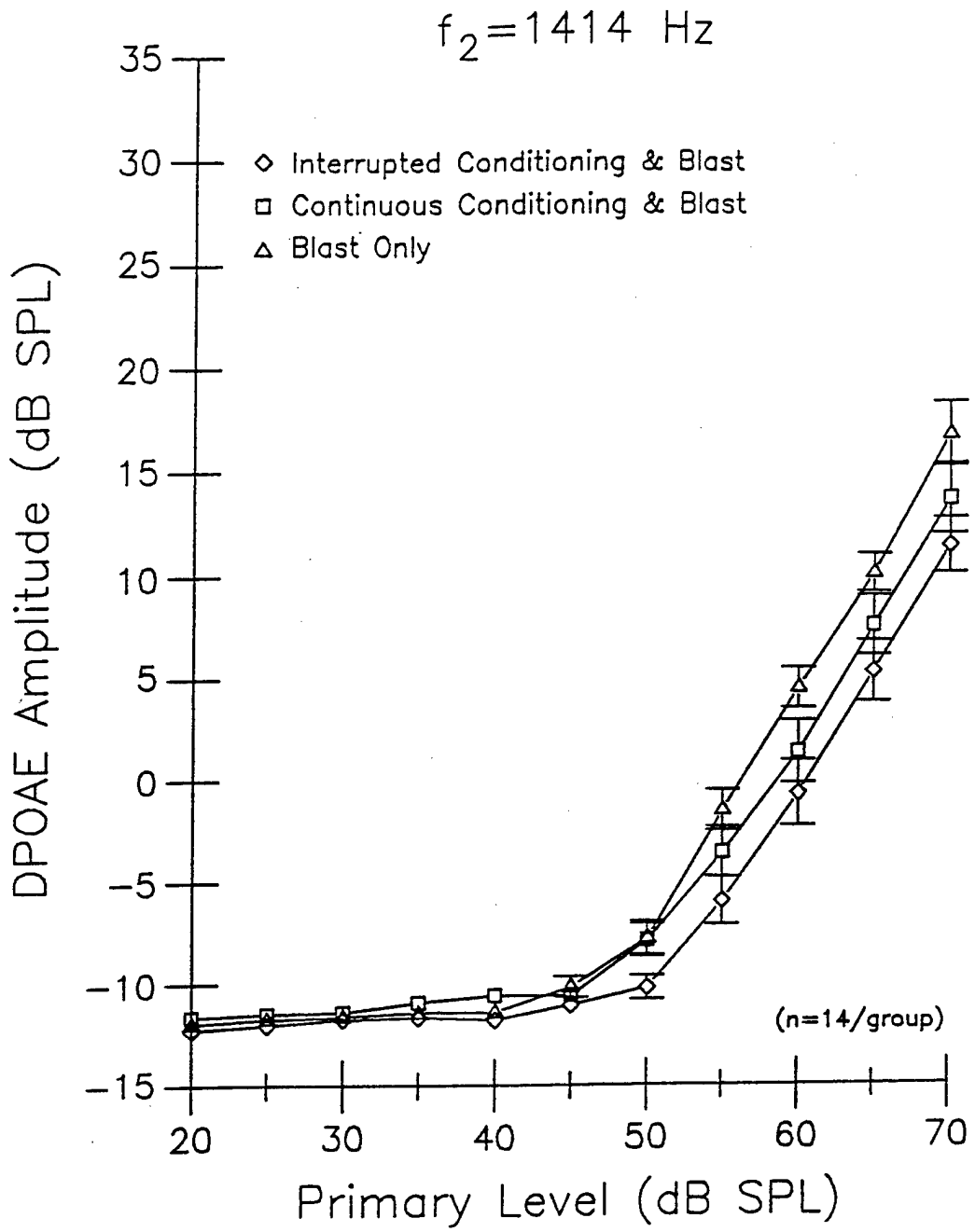

Figure 4c The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 1414 Hz. For additional information, see legend for Figure 3a.

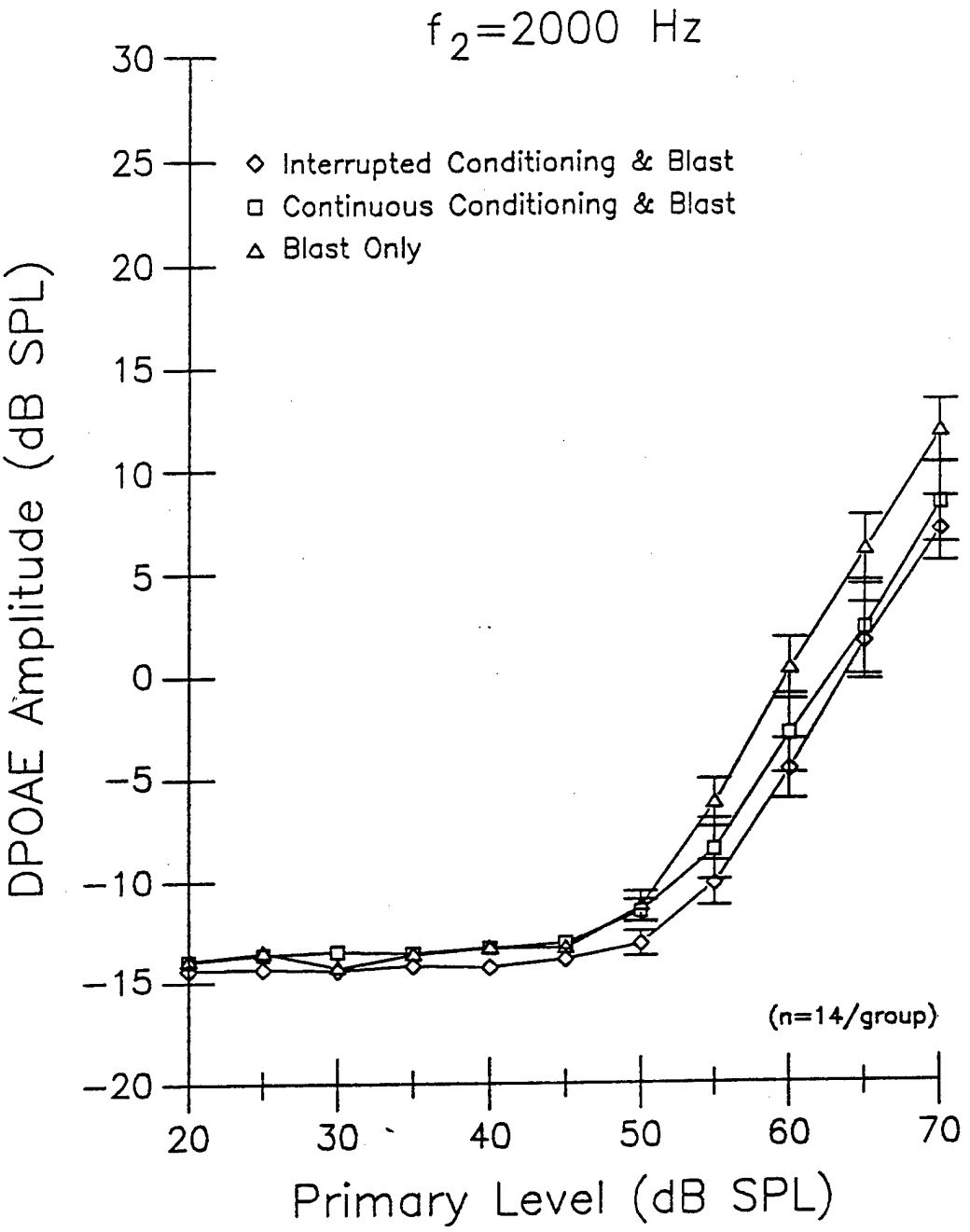

Figure 4d The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 2000 Hz. For additional information, see legend for Figure 3a.

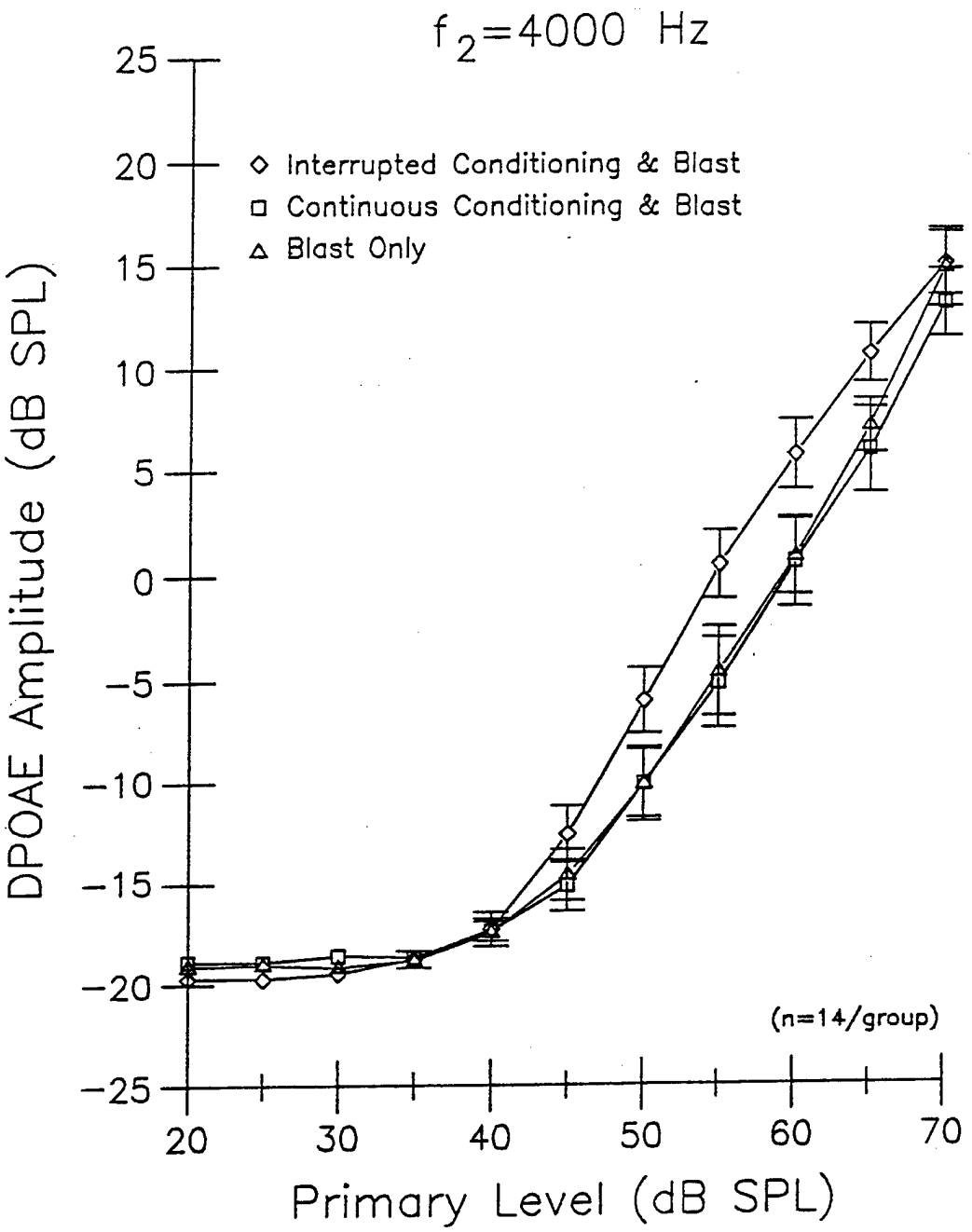

Figure 11f The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 4000 Hz. For additional information, see legend for Figure 3a.

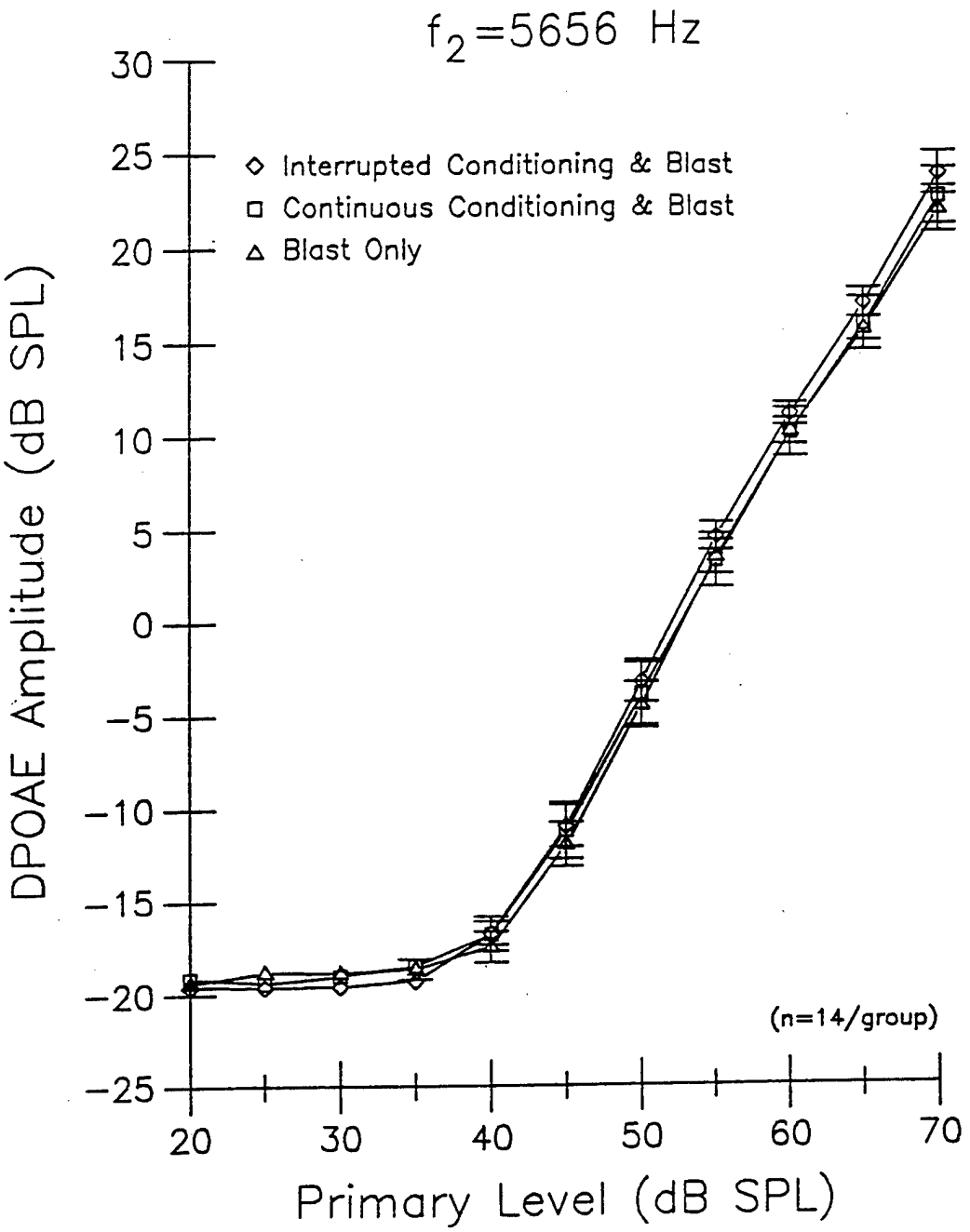

Figure 4g The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 5656 Hz. For additional information, see legend for Figure 3a.

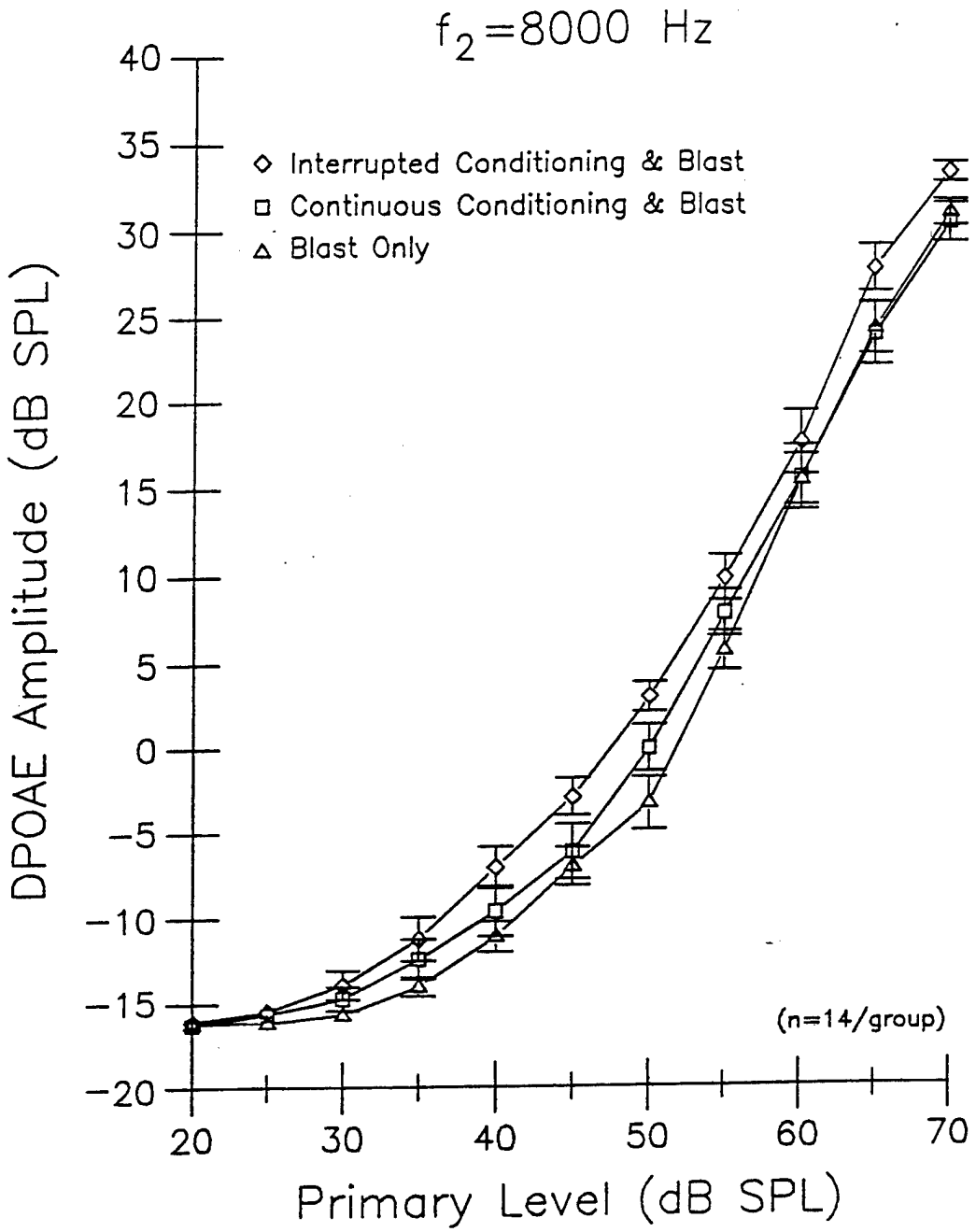

Figure 4h The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 8000 Hz. For additional information, see legend for Figure 3a.

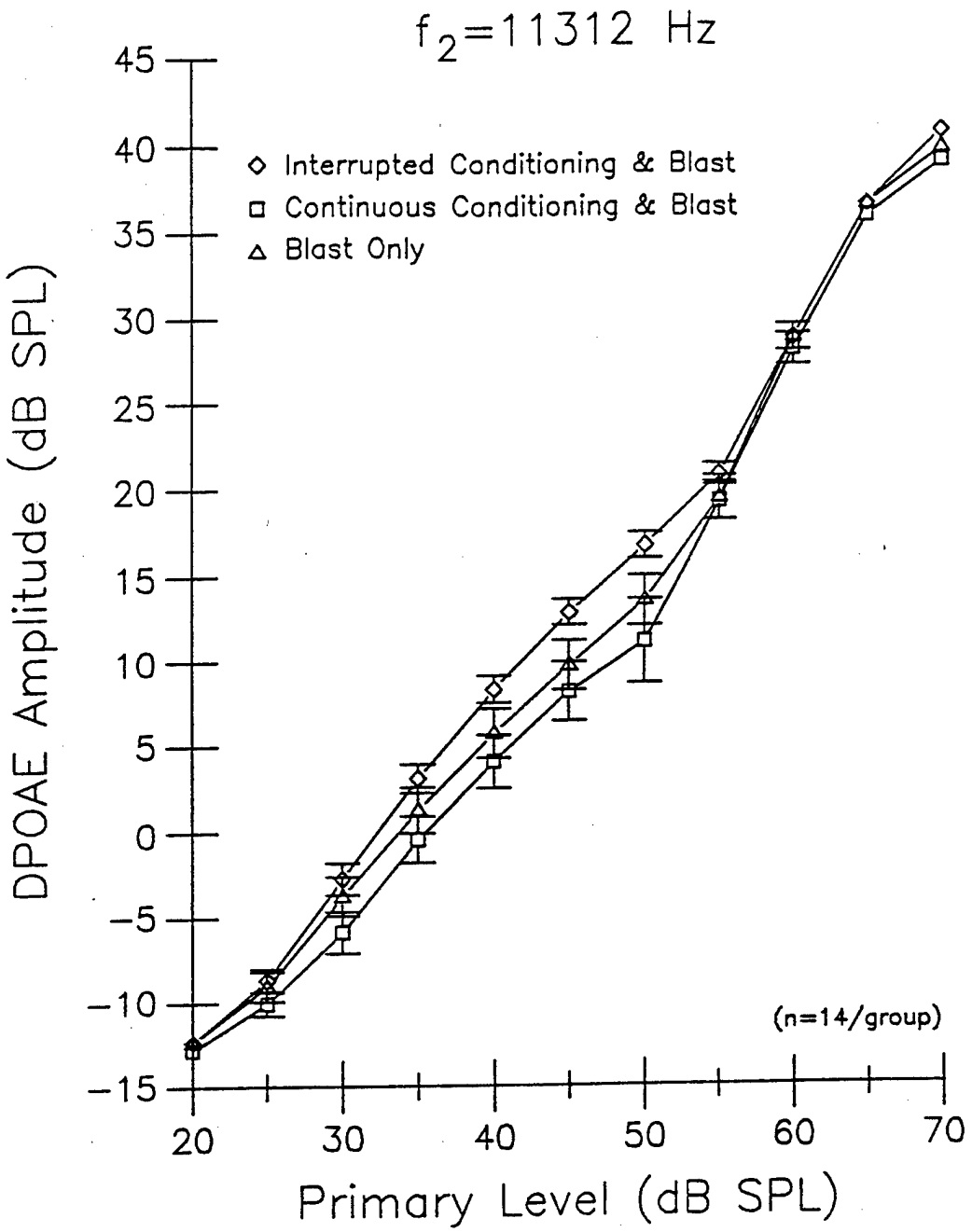

Figure 41 The effect of the traumatizing exposure on DPOAE responses of unconditioned and sound conditioned animals at f_2 = 11312 Hz. For additional information, see legend for Figure 3a.

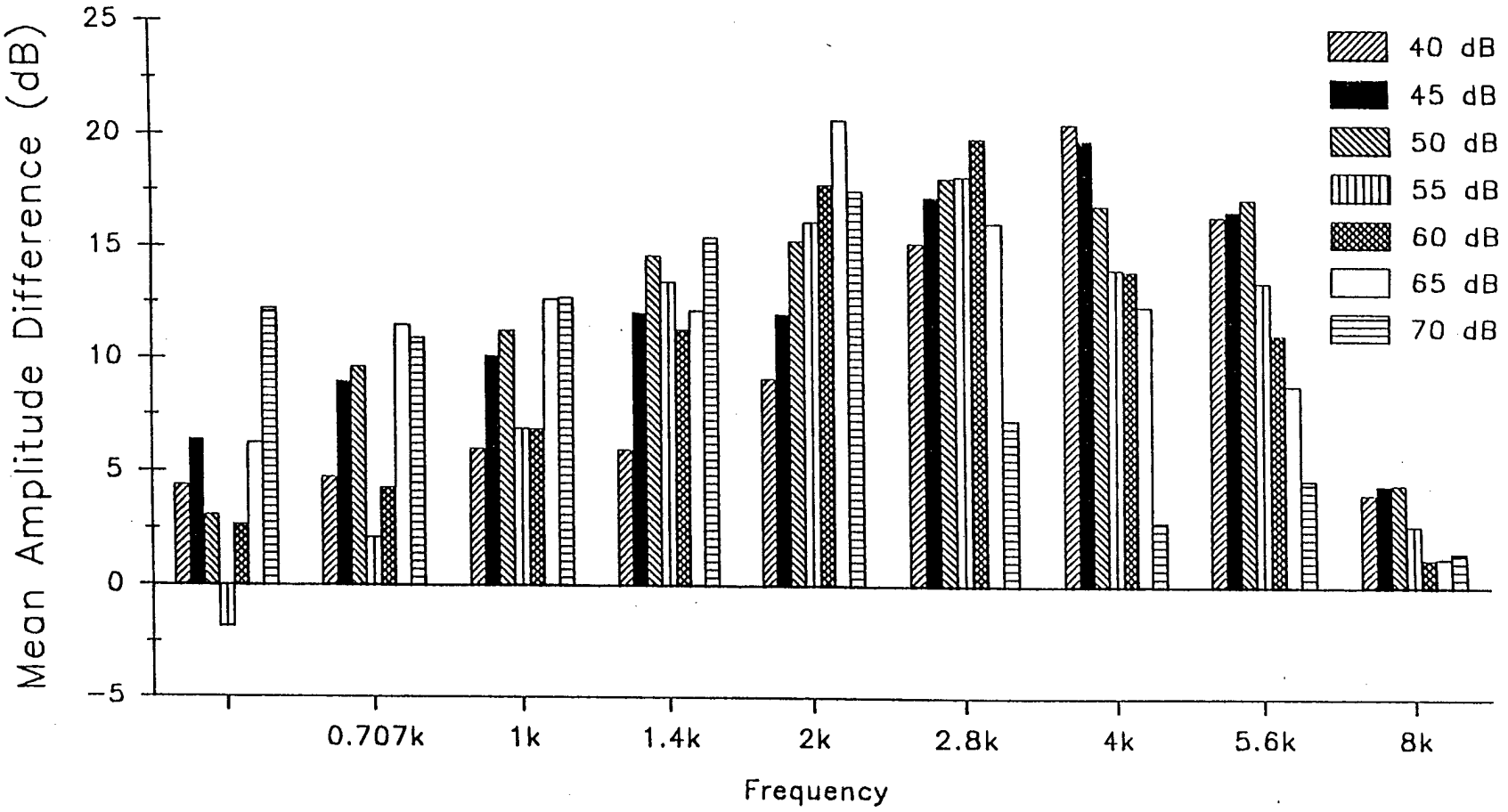

Figure 5a The effect of the traumatizing noise on normal DPOAE responses. Data are presented as the mean amplitude differences between the Aged Normal Group and the Blast Only Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

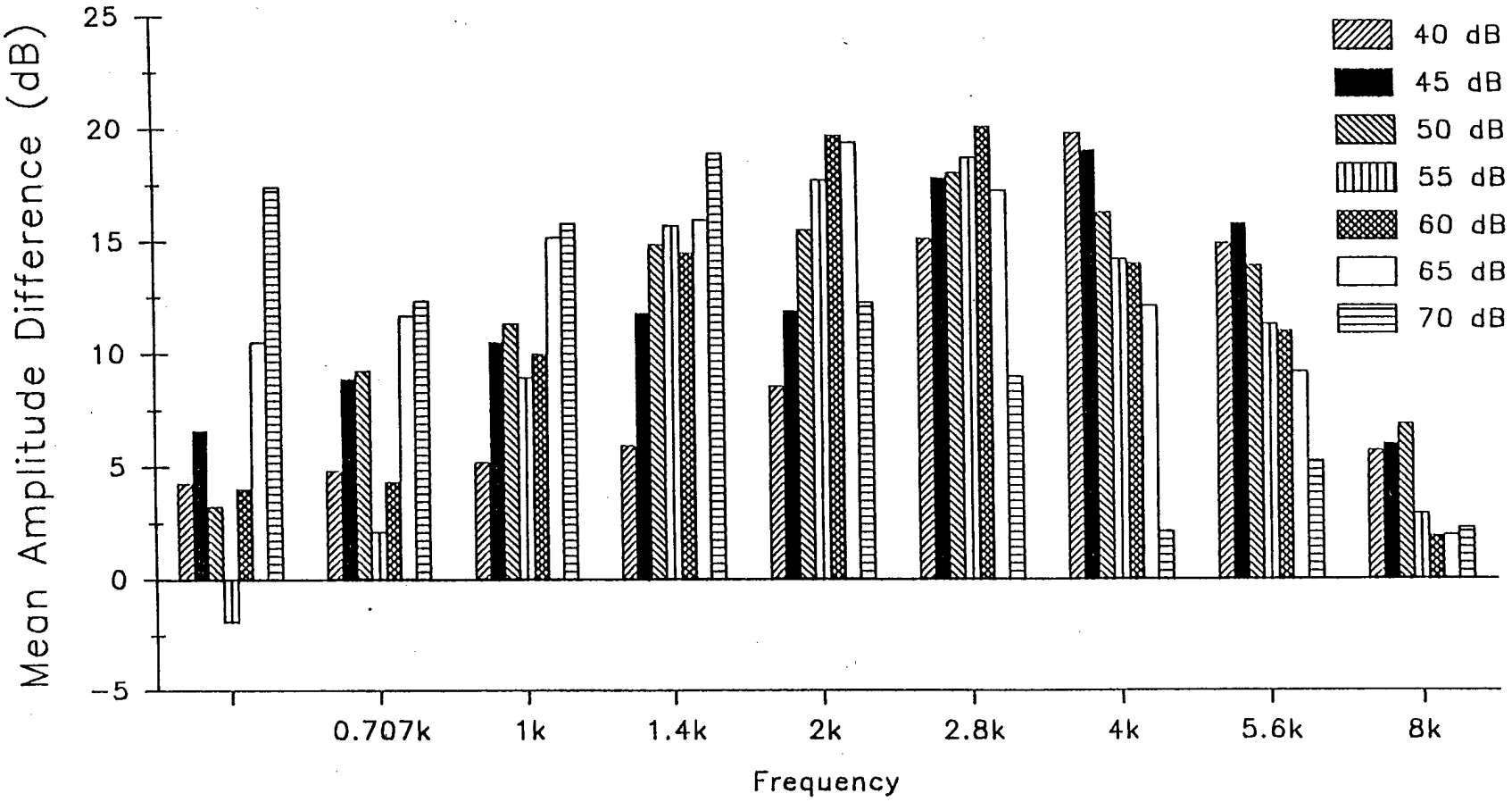

Figure 5b The effect of the continuous conditioning/traumatizing noise exposure combination on normal DPOAE responses. Data are presented as the mean amplitude differences between the Aged Normal Group and the Continuous Conditioning then Blast Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

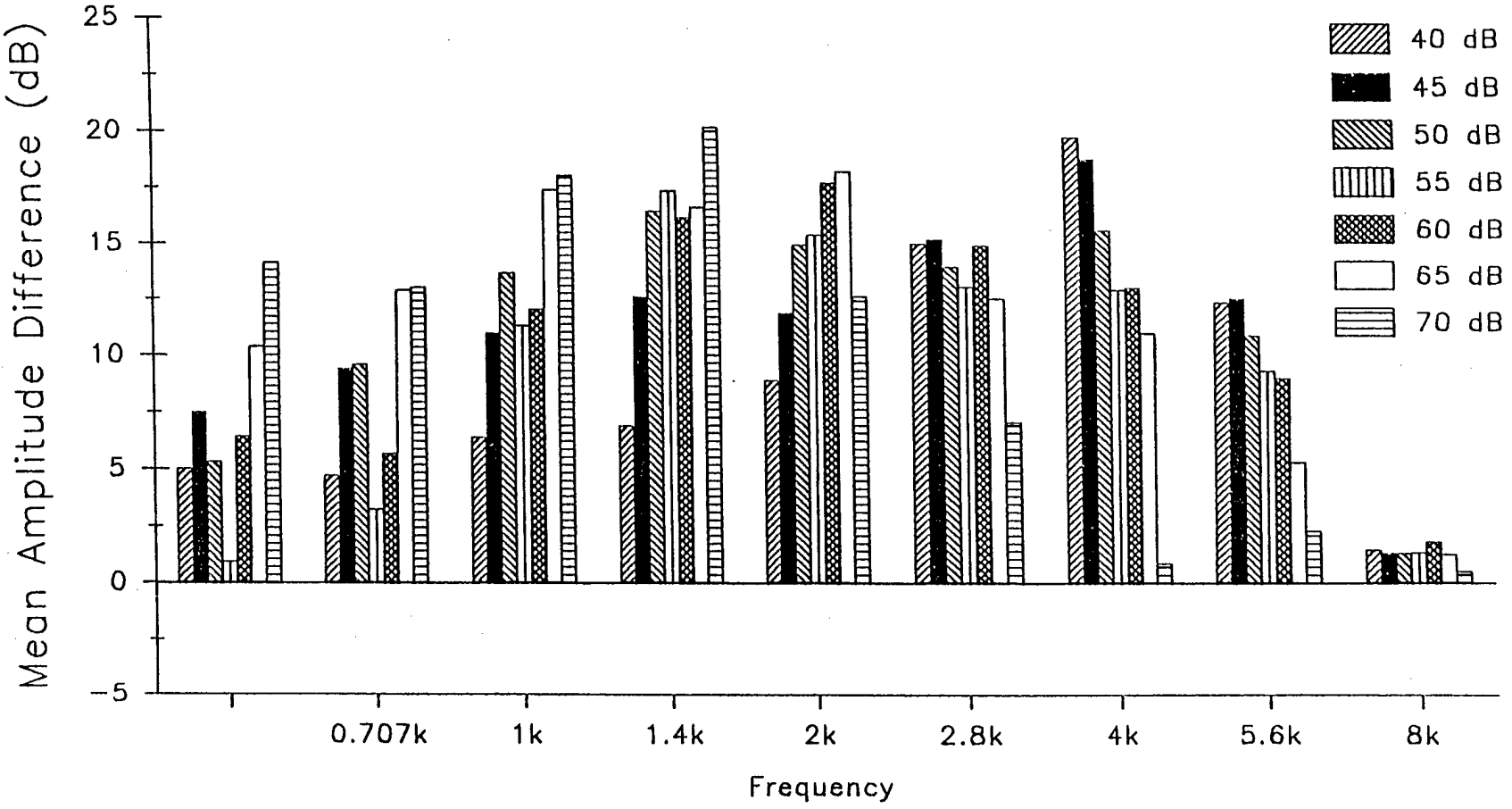

Figure 5c. The effect of the interrupted conditioning/traumatizing noise exposure combination on normal DPOAE responses. Data are presented as the mean amplitude differences between the Aged Normal Group and the Interrupted Conditioning then Blast Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

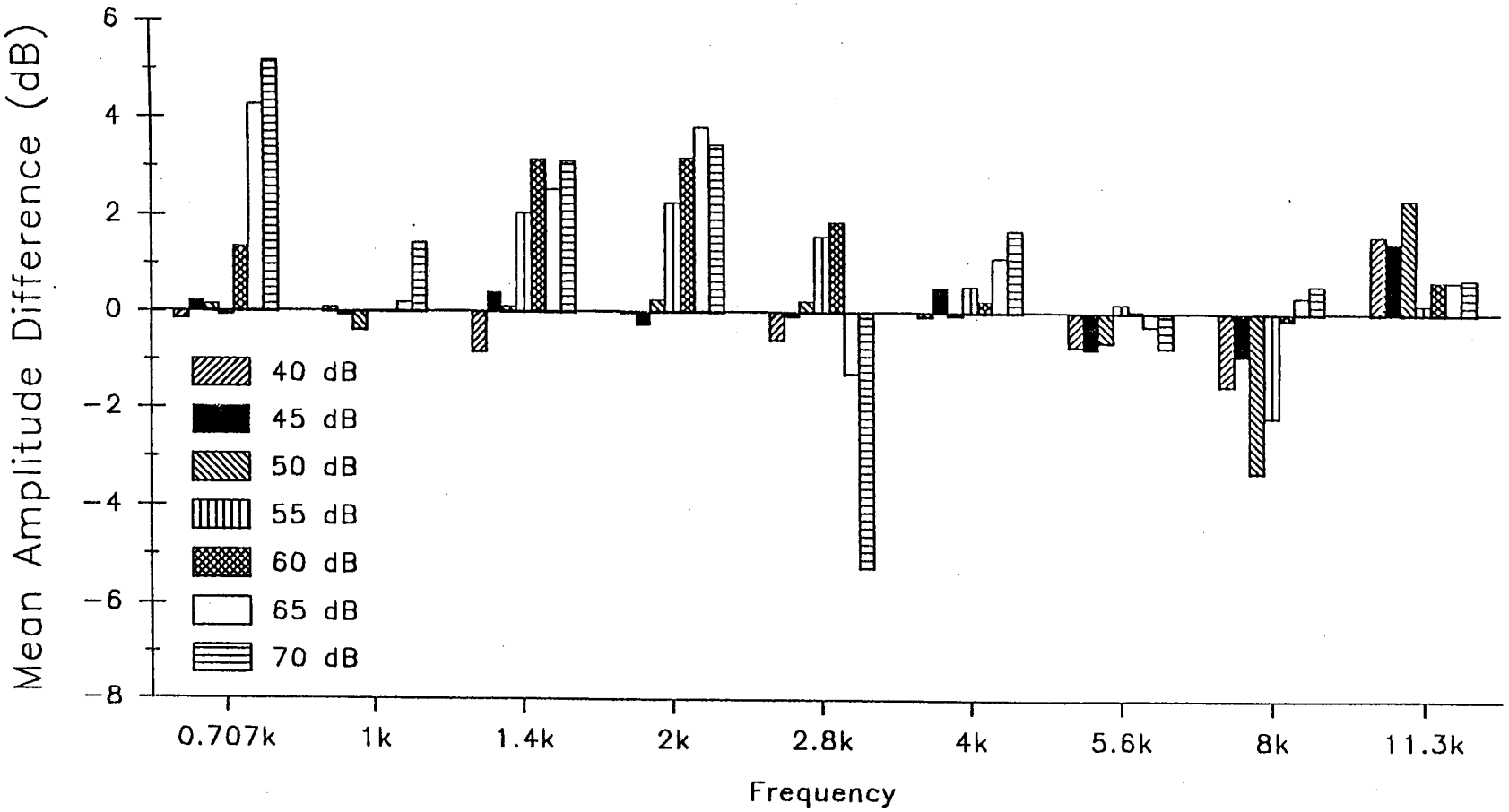

Figure 6a. Comparison of the effect of the traumatizing noise on the DPOAE responses of the unconditioned vs. the continuous sound conditioned groups. Data are presented as the mean amplitude differences between the Blast Only Group and the Continuous Conditioning then Blast Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

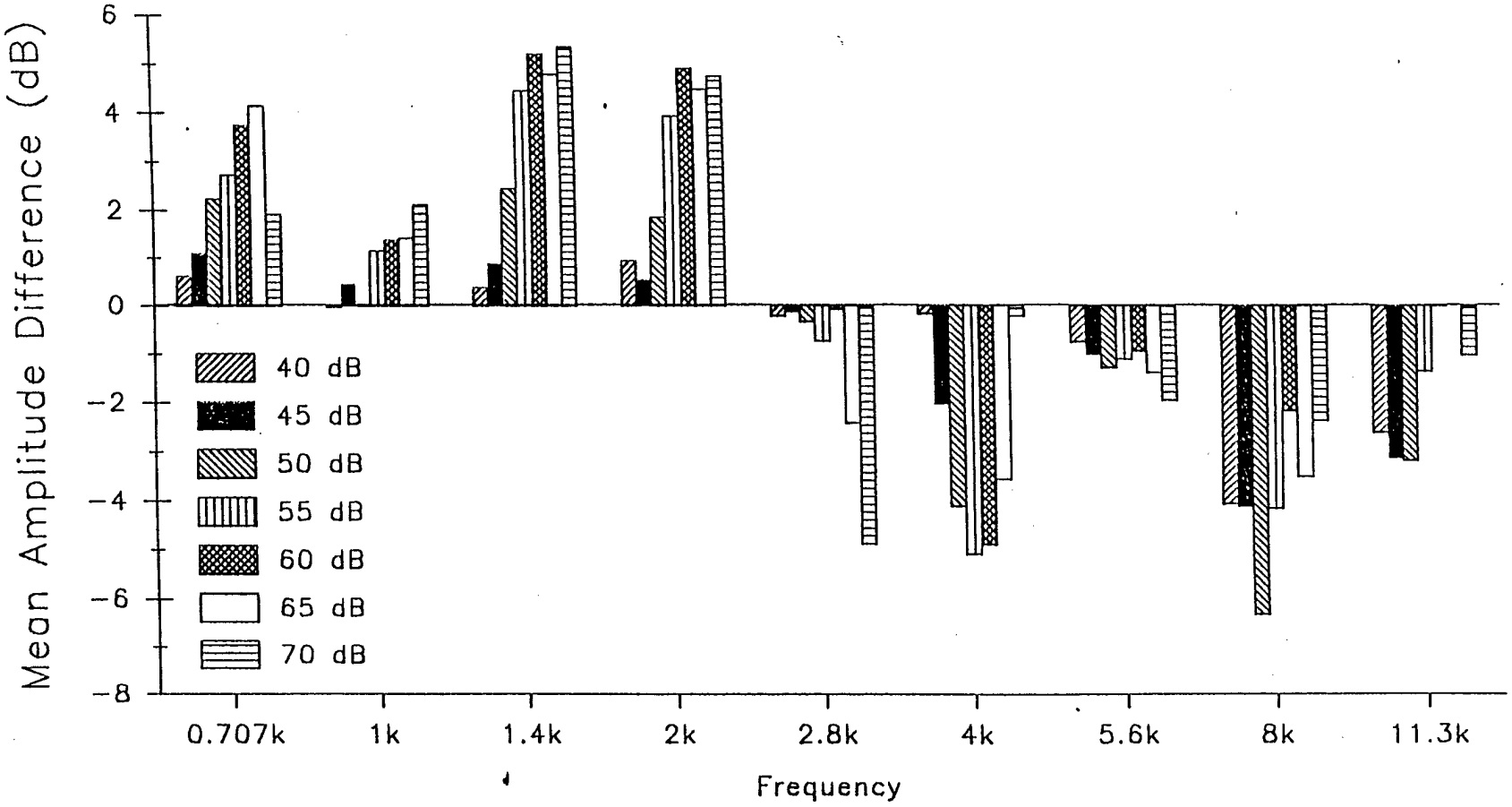

Figure 6b Comparison of the effect of the traumatizing noise on the DPOAE responses of the unconditioned vs. the interrupted sound conditioned groups. Data are presented as the mean amplitude differences between the Blast Only Group and the Interrupted Conditioning then Blast Group (in dB) plotted as a function of frequency (0.707-11.312 kHz) for several different primary intensity levels (40-70 dB SPL; inset).

APPENDIX # 7
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Failure to detect an ATP-evoked current response in outer hair cells of rat cochlea

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Abstract

Increasing evidence suggests that adenosine 5' triphophate (ATP) may act as a neurotransmitter and/or modulator in the mammalian cochlea. In our laboratory, it was observed that there are differences in pharmacology of the ACh receptor in outer hair cells (OHCs) of rat compared to those of guinea pig (Chen et al., 1996a). During those studies, it was also noted that there may be a difference in the effect of ATP on OHCs of rat compared to the effect on OHCs of the guinea pig. The purpose of the present study was to compare the ATP response recorded from OHCs of guinea pig to that of rat using the whole-cell configuration of the patch-clamp technique. Results show that the extracellular application of 100 μM ATP or ATP-γ-S induced a large inward current in OHCs of guinea pig recorded at -60 and -100 mV, while no response was detected in rat at the same concentration. However, in the majority of these same rat and guinea pig OHCs, 100 μM ACh did induce an inward current at -100 mV and an outward current at 0 mV. When N-methyl-glucamine was substitued for K^+ in the pipette solution in order to unmask any ATP-evoked inward current, no ATP-induced current was detected in rat OHCs. Nor was any response produced in rat or guinea pig OHCs by either adenosine, adenosine 5'-monophosphate or adenosine 5'-diphosphate at 100 μM. ATP did, however, produce a very large inward current in Deiter's cells isolated from both rat and guinea pig,. Results therefore suggest that there may be no, or very few, functional, ATP-gated cation-selective channels in OHCs of rat cochlea in contrast to the apparent large number in OHCs of guinea pig cochlea.

Key words: ATP; Receptor; Cochlea; Ion channel; Voltage-clamp

1. Introduction

Adenosine 5'-triphosphate (ATP) has been suggested to subserve both neurotransmitter and neuromodulator functions in a number of neuronal systems (see review: Burnstock, 1990). At least five types of ATP receptors have been identified based on physiological and pharmacological characteristics (Kennedy and Leff, 1995). Both ligand gated (ionotropic) and G protein coupled (metabotropic) ATP receptors have been cloned (Lustig et al., 1993; Webb et al., 1993; Brake et al., 1994; Filtz et al., 1994; Valera et al., 1994; Housley et al., 1995; Surprenant et al., 1995; Collo et al., 1996).

Increasing evidence has shown that ATP may act as a neurotransmitter or neuromodulator in the mammalian cochlea (Bobbin, 1996; Eybalin, 1993). In vivo experiments of Bobbin and Thompson (1978) and Kujawa et al., (1994a) show that ATP and related agonists (e.g., ATP-γ-S) placed in the perilymph compartment have significant effects on the compound action potential of the auditory nerve (CAP), cochlear microphonics (CM), summating potential (SP), as well as distortion product otoacoustic emissions (DPOAEs) in guinea pigs. In subsequent studies, it was further demonstrated that the ATP antagonists, cibicron blue, basilen blue and suramin, exert profound influences on sound-evoked responses, suggesting that there exists endogenous release of ATP that affects cochlear function via the P_2 subtype of purinergic receptors (Kujawa et al., 1994b).

In vitro studies indicate that ATP activates receptors on several cells in the cochlea. ATP activates an ionotropic receptor on isolated OHCs that induces inward cation currents (Na^+, Ca^{2+}) and depolarizes the cell membrane (Ashmore and Ohmori, 1990; Housley et al., 1992; Ikeda et

al., 1991; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et. al., 1990; Nilles et al., 1994). ATP also activates a metabotropic receptor that increases intracellular free Ca^{2+} in these cells (Ashmore and Ohmori 1990; Ikeda et al., 1991; Shigemoto and Ohmori, 1990). Additional evidence for a metabotropic receptor mechanism was obtained by Niedzielski and Schacht (1992) which showed an increase in ionositol phosphate accumulation in guinea pig organ of Corti following incubation with ATP agonists (see also: Ogawa and Schacht, 1993, 1994, 1995). A heterogeneous modulation of L-type Ca^{2+} channel currents by ATP in OHCs of guinea pig cochlea was reported (Chen et al., 1995a). Activation of ATP receptors increases intracellular Ca^{2+} levels in IHCs (Dulon et al., 1991), Deiters' cells (Dulon et al., 1993) and Hensen's cells (Dulon et al., 1993) and depolarizes both Deiters' cells (Dulon, 1995) and IHCs (Dulon et al., 1991). Other evidence demonstrates that ATP induces changes in the cells of the stria vascularis (Suzuki et al., 1995; Wangemann, 1995), and the origin of the endogenous ATP that acts on these receptors may be the marginal cells in the stria vascularis (White et al., 1995). ATP occurs in high concentrations in endolymph (Munoz et al., 1995a), and when placed in the endolymph, ATP induces large changes in cochlear function (Munoz et al., 1995b). Thus ATP appears to have an important paracrine and/or autocrine role in the cells of the cochlea.

Recently, Chen et al (1996a) observed that there are pharmacological differences in the cholinergic receptor of OHCs isolated from guinea pig cochlea when compared to those isolated from rat cochlea. Preliminary results indicated that ATP did not evoke a current response in OHCs isolated from rat cochlea. No known studies have examined the effect of extracellular ATP on the OHCs of rat cochlea. Therefore, the present study compares the effects of ATP on ionic currents recorded from guinea pig OHCs to those recorded from rat OHCs using the whole-cell

configuration of the patch-clamp technique. Preliminary results have been presented (Chen et al., 1996b).

2. Methods

2.1. Isolation of OHCs and Deiters' cells

OHCs and Deiters' cells from 37 pigmented guinea pigs, 56 Sprague Dawley rats and 9 pigmented Long Evans rats were isolated as described previously (Zajic & Schacht, 1987; Chen et al., 1995a; 1995b). Unless stated otherwise, the data presented from rat is from the Sprague Dawley strain. Animals were anesthetized with pentobarbital (30 mg/kg, i.p. for guinea pigs and 50 mg/kg, i.p. for rats), decapitated, and the bulla separated and placed in a modified Hank's balanced saline (HBS). The bone surrounding the cochlea was removed, and the organ of Corti was placed in 200 µl of HBS containing collagenase (1 mg/ml, Type IV, Sigma) for 5 min. The cells were then transferred into the dishes containing a 150 µl drop of HBS using a microsyringe and stored at room temperature. The length of each cell was measured with a calibrated reticulum prior to recording.

2.2. Whole-cell voltage clamp

Single dispersed rat or guinea pig OHCs and Deiters' cells were voltage or current clamped using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) with an Axopatch-1D patch-clamp amplifier (Axon Instruments). Patch electrodes were fabricated from

borosilicated capillary tubing (Longreach Scientific Resources) using a micropipette puller (Sutter Instrument Co.), and fire polished on a microforge (Narashige Scientific Instrument Lab.) prior to use. Membrane currents were filtered at 5 KHz (- 3dB) using a four-pole low-pass Bessel filter digitized with a 12-bit A/D converter (DMA Interface, Axon Instruments), and stored for off-line analysis using a pentium microcomputer. Voltage paradigms were generated from a 12-bit D/A converter (DMA Interface, Axon Instruments) using pClamp software (Axon Instruments). After establishment of the whole-cell configuration, series resistance and cell capacitance compensation were carried out prior to recording with 80 % series resistance compensation normally applied. No substraction of leakage current was made.

2.3. Solutions

The HBS utilized for isolating cells and perfusing the bath contained (mM): 145 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES and 10 glucose. The HBS solution was adjusted to a pH of 7.40 with NaOH and to 300 mOsm/kg H_2O with sucrose. The K^+ internal solution contained (mM): 140 KCl, 0.5 $MgCl_2$, 5 HEPES, 11 EGTA, 0.1 $CaCl_2$, 2 Na_2ATPand 0.1 Na_2GTP. The N-methyl-glucamine (NMG^+) internal solution contained (mM): 120 NMG^+, 35 TEA-Cl, 11 EGTA, 0.5 $CaCl_2$, 10 HEPES, 4 MgATP, 0.1 Na_2GTP and 5 sucrose. Both internal solutions were adjusted to a pH of 7.35 with HCl and had a osmolality of 284 mOsm/kg H_2O with sucrose. The ATP and its related agonists tested were freshly prepared at desired concentrations in the HBS external solution. The drugs used were: ATP, adenosine 5'-0-(3-thiotriphosphate) tetralithium salt (ATP-γ-S), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine (Adeno) and acetylcholine (ACh). All the drugs were purchased from Sigma

Chemical Company (St. Louis, MO). All the drug solutions were delivered from a U-tubing system as described previously (Erostegui et al., 1994; Chen et al., 1995a; 1995b). All experiments were conducted at room temperature (22 ~ 24 °C). The care and use of the animals reported on in this study were approved by the Animal Care and Use Committees of Louisiana State University Medical Center.

3. Results

3.1. Effects of ATP on rat and guinea pig OHCs.

Our laboratory previously reported (Chen et al., 1996a) that the average length of the rat OHCs studied were shorter (mean, 32 μm) than those from guinea pig (mean, 48 μm) , and that there was no difference in the resting membrane potential of the rat OHCs (mean, -66 mV) compared to guinea pig (about -69 mV). As observed previously by our laboratory (Kujawa et al., 1994a; Chen et al., 1995a; 1995b) and others (Nakagawa et al., 1990; Housley et al., 1992; Mockett et al., 1994), an extracellular application of ATP induced an inward current in the majority of OHCs (n = 53) from guinea pig cochlea that were tested. In contrast, application of ATP (100 μM, n = 54; 1 mM, n = 4) did not produced any detectable current in OHCs from rat. Figure 1 illustrates current-voltage (I-V) relationships of ATP evoked current in OHCs from rat (Fig. 1A) and guinea pig (Fig. 1B). A voltage-ramp was applied between -150 mV and +60 mV (0.75 mV/ms) from a holding potential of -60 mV. As shown, 100 μM ATP did not elicit a detectable current in an OHC of rat (Fig. 1A). In contrast, 100 μM ATP did induce a response in an OHC of guinea pig (Fig. 1B).

3.2. Effects of ion substitution.

The ATP-induced current is a cation current that is usually directed inward at potentials negative to 0 mV. The current is carried by Na^+ and Ca^{2+}. There is the possibility that a Ca^{2+} activated, outward K^+ current maskes the ATP-evoked inward current in rat OHCs. Therefore, to prevent an outward K^+ current, NMG^+ was substituted for K^+ in the pipette. However, even under these conditions, ATP (100 μM) failed to induced a detectable inward current in rat OHCs (n = 4).

3.3. Effects of ATP analogues.

Due to the fact that ATP breakdown is relatively fast, we employed a more slowly hydrolysible ATP analog, ATP-γ-S, to test whether there is an ATP-induced current in rat OHCs. As shown in Figure 2B, extracellular application 100 μM ATP-γ-S did not induce a detectable current response in OHCs isolated from rat (n = 6), but did elicit an inward current in OHCs (n = 4) isolated from guinea pig (Fig. 2D). It is possible that the ionotropic ATP receptor on rat OHCs may have a different pharmacology than the receptor on guinea pig OHCs. Therefore, we tested the effects of ADP, AMP and adenosine. As shown in Figure 3, these agents did not induce any detectable current response in OHCs from either guinea pig or rat (guinea pig OHCs: ADP, n = 9; AMP, n = 7; adenosine, n = 12; rat OHCs: ADP, n = 8; AMP, n = 6; adenosine, n = 6). It is interesting to note the ATP-induced outward current in Figure 1B, and the ATP-induced delayed outward current at 0 mV in Figure 3 B. A possible explanation is that an ATP-induced increase in internal Ca^{2+} subsequently activates a Ca^{2+} dependent outward K^+ current.

3.4 Effects of ACh on OHCs and of ATP on Deiters' cells.

Another explanation for ATP not inducing a detectable current is that the isolation procedure was generally distructive to ligand receptors. To test this possibility, 100 μM ACh and 100 μM ATP were applied to both OHCs isolated from rat and guinea pig. Figure 4 shows that 100 μM ACh evoked an inward current at -100 mV and an outward current at 0 mV in both rat (n = 49 out of 51cells tested) and guinea pig (n = 56 out of 58 cells tested) OHCs. ATP (100 μM) did not evoke a current response in 40 of the rat OHCs responding to ACh, but ATP (100 μM) did evoke a current response in 45 of the 47 guinea pig OHCs responding to ACh. In addition, ATP (10 μM)-evoked a large inward current that was rapidly desensitized in Deiters' cells isolated from the cochleae of both rat (n = 7) and guinea pig (n = 7; Fig. 5). In vivo, Deiters' cells are attached to the OHCs and, although their ionotropic ATP receptors may be slightly different, one would expect that any degradation induced by the isolation procedure would affect both receptors to the same extent.

3.5 Effects of pigmentation or strain

To test for a relationship between the ATP evoked current response and the degree of pigment in the rat or strain of rat, OHCs were obtained from 9 pigmented rats (Long Evans). ATP (100 μM) did not evoke a detectable current in 9 of the OHCs tested, but 7 of these cells did demonstrate a typical current change in response to the application of 100 μM ACh. Two cells did not respond to either ATP or ACh.

4. Discussion

To date, investigators have reported that ATP activates an ionotropic receptor that induces an inward cation current in OHCs isolated from the guinea pig (Ashmore and Ohmori, 1990; Chen et al., 1995a, 1995b; Housley et al., 1992; Ikeda et al., 1991; Kakehata et al., 1993; Kujawa et al., 1994b; Nakagawa et. al., 1990; Nilles et al., 1994; Shigemoto and Ohmori, 1990). The present study used external applications of ATP and ATP agonists to test the hypothesis that an ionotropic ATP receptor is present on OHCs of the rat cochlea. The results failed to detect an ATP-induced inward current in rat OHCs. This may be an indication that ionotropic receptors are not present on OHCs of the rat cochlea, or if present, they are not functional.

The lack of an ionotropic response to ATP application may be due to physical or chemical damage to the ATP receptors. Investigators have suggested that the ionotropic receptor on OHCs is located near the stereocilia portion of the cells (Housley et al., 1992; Mockett et al. 1994 and 1995). Stereocilia are delicate structures and are easily damaged during the physical isolation of the OHCs. Thus the lack of response to ATP may be due to some physical alteration in the receptors located near or on damaged stereocilia. Yet, this does not seem to be the case with guinea pig OHCs, which appear to exhibit ATP-induced currents even in OHCs with damaged stereocilia (unpublished observations). Alternatively, the receptor may have been damaged by collagenase or another chemical released from the tissue during the isolation proceedure. However, this seems unlikely given the large inward currents evoked by ATP in Deiters' cells isolated from the same rat cochlea, indicating that Deiters' cells' ATP receptors were not chemically altered into a non-functioning state.

What the results may indicate is a functional difference between rat and guinea pig OHCs. At present, one can only speculate about this possibility. For example, Chen et al., (1995b) observed that exposing guinea pigs to low levels (64 dB SPL) of chronic (10 days) noise modified the magnitude of the ATP-induced current observed in OHCs, increasing it in long OHCs and decreasing it in short OHCs. In a companion study, Skellett et al., (1996) demonstrated that this same noise exposure resulted in a significant reduction in DPOAEs. These results suggest that ATP receptors are not static, but rather adapt their response characteristics in response to varying physiological or pathophysiological events in the cochlea. Thus, ATP receptor proteins in the cochlea may behave similarly to other receptors by being up- or down-regulated or modulated during various physiological and environmental conditions. The rat is less sensitive than guinea pig to the effects of intense sound (Borg et al., 1995). Thus, the ionotropic ATP receptor on OHCs of the rat may be modulated or down-regulated to a great extent. This mechanism may desensitize the rat to damaging effects of intense sounds in its environment.

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Figure legends:

Fig. 1. A: Current-voltage (I-V) relationships recorded from a rat outer hair cell (OHC) in the absence and presence of 100 μM ATP. B: I-V curves recorded from a guinea pig OHC in the absence and presence of 100 μM ATP. The voltage command was ramped from -150 mV to +60 mV (0.5 mV/ms). Membrane potential was held at -60 mV.

Fig. 2. Example of the absence of a detectable current response from a rat OHC at -100 mV during extracellular application of 100 μM ATP (A) or from another rat OHC to 100 μM ATP-γ-S (B) . Examples of guinea pig OHCs elicited a current response at -100 mV during extracellular application of 100 μM ATP (C) or from another OHC 100 μM ATP-γ-S (D). Current was elecited by a 70 ms hyperpolaring step to -100 mV from a holding potential of -60 mV with steps presented every second. Drug was applied for 20 or 30 step presntations and recovery but not wash-out is shown.

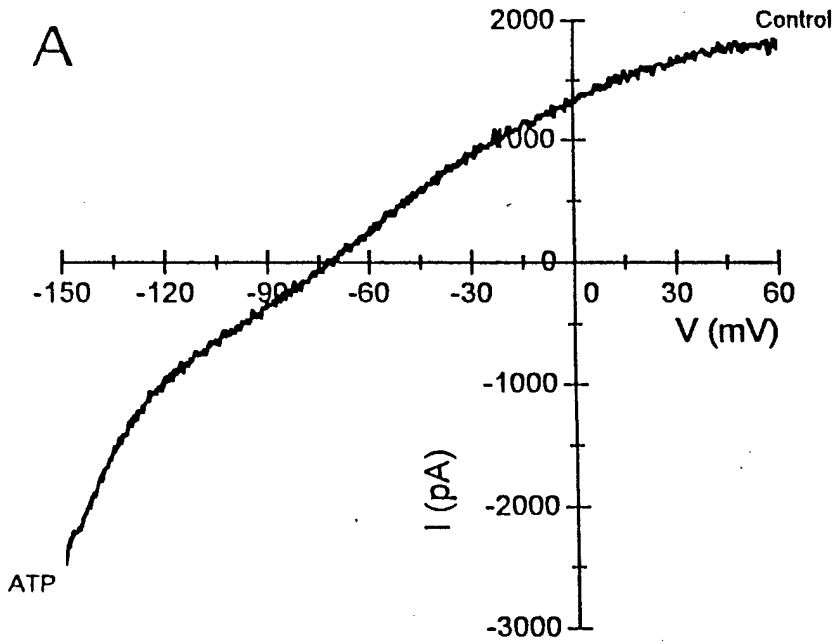
Fig. 3. Examples of the current responses of a rat OHC (A) and a guinea pig OHC (B) to the application of adenosine, AMP, ADP, ATP and ACh. Voltage protocol and trace construction was the same as in Fig. 3. Drug application is indicated by the bars.

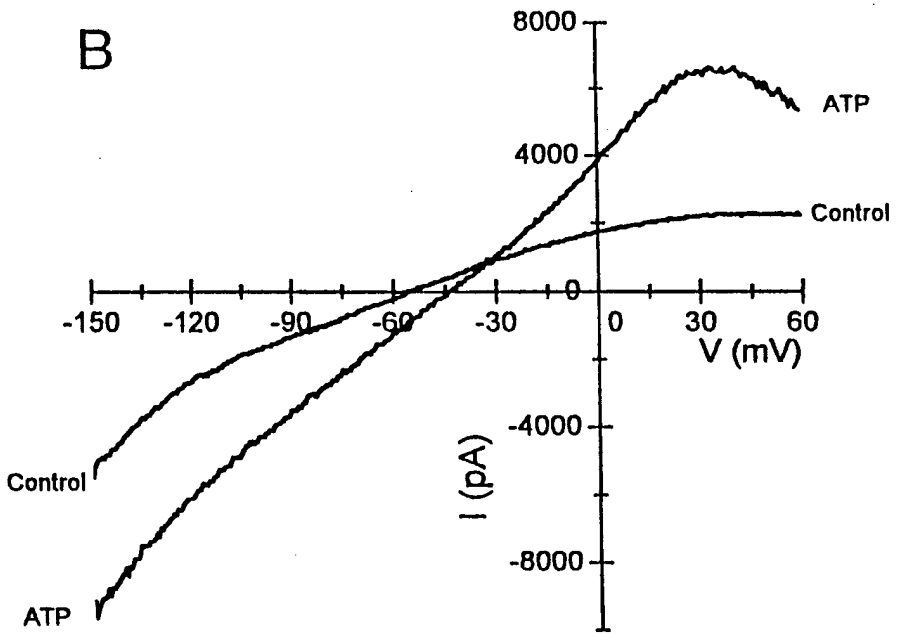
Fig. 4. A: Superimposed step-evoked currents recorded from a rat OHC in the absence (control) and presence of 100 μM acetylcholine (ACh), and recorded from the same cell in the absence (control) and presence of 100 μM ATP. B: Superimposed step-evoked currents recorded from a guinea pig OHC in the absence (control) and presence of 100 μM ACh, and recorded from the same cell in the absence (control) and presence of 100 μM ATP. In both A and B the dashed line is the zero current level. C: Time course of the ACh and ATP-induced responses at -100 mV and 0 mV in a rat OHC. D: Time course of the ACh and ATP-induced responses at -100 mV and 0 mV in a guinea pig OHC. Traces were constructed utilizing the protocol shown for A and B from the current value measured 25ms after the onset of the hyperpolaring step to -100 mV and 5ms after the onset of the depolarizing step to 0 mV from a holding potential of -60 mV with steps presented every second. Drug was applied for 20 step presentations. Wash-out is not shown.

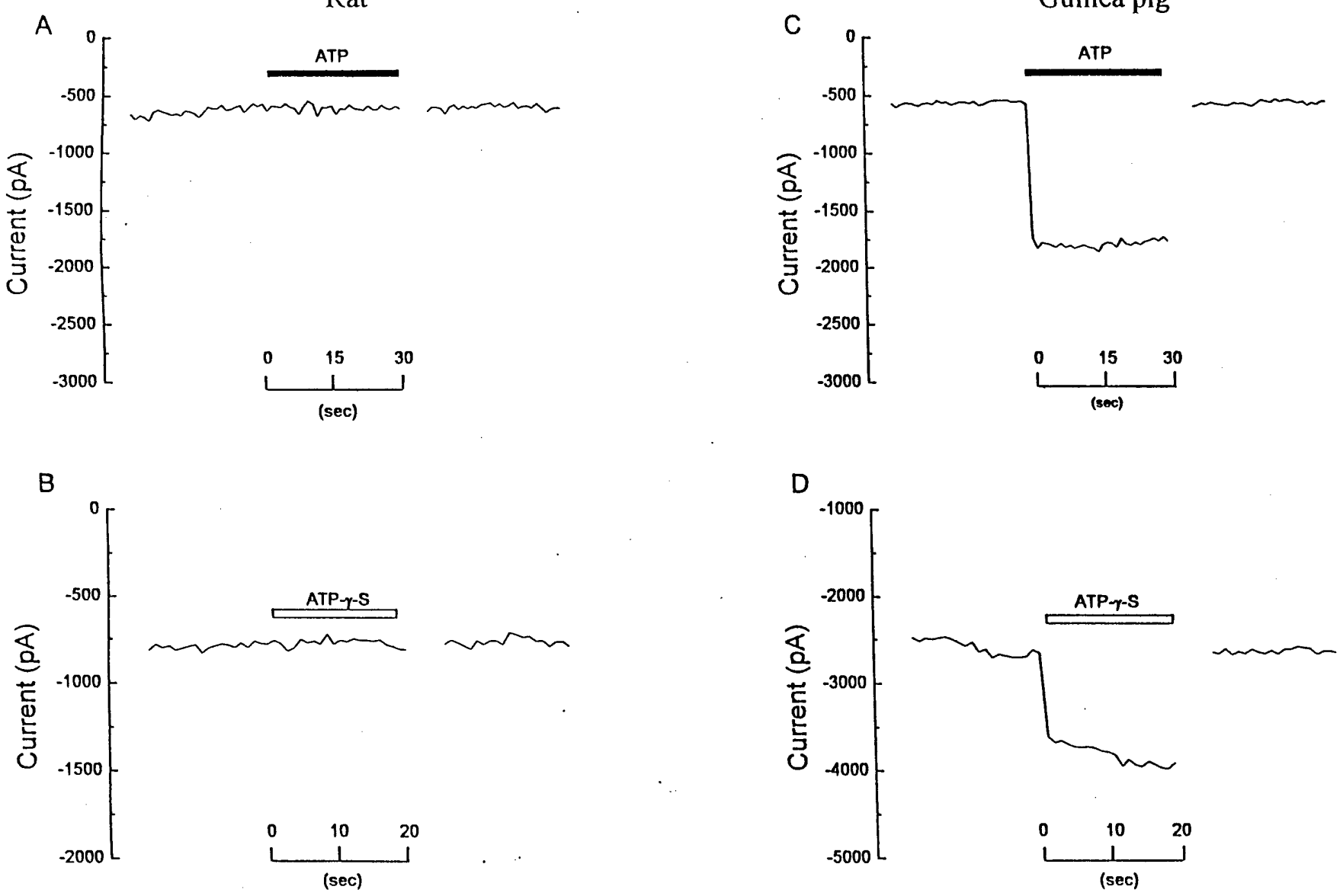
Fig. 5. Examples of an ATP-induced inward current response obtained from a rat Deiters' cell (A) and a guinea pig Deiters' cell (B) at -70 mV. Current-voltage (I-V) relationships were recorded in the absence and presence of 10 μM ATP. The voltage command was ramped from -150 mV to +60 mV (0.5 mV/ms) and the ramp was presented at the rate of one every second. Membrane potential was held at -60 mV. Traces were constructed from the current evoked by the ramp utilizing the current value obtained at -70 mV.

Rat

Guinea pig






Rat
Guinea pig
A
B
C
D
ATP
ATP-γ-S
Current (pA)
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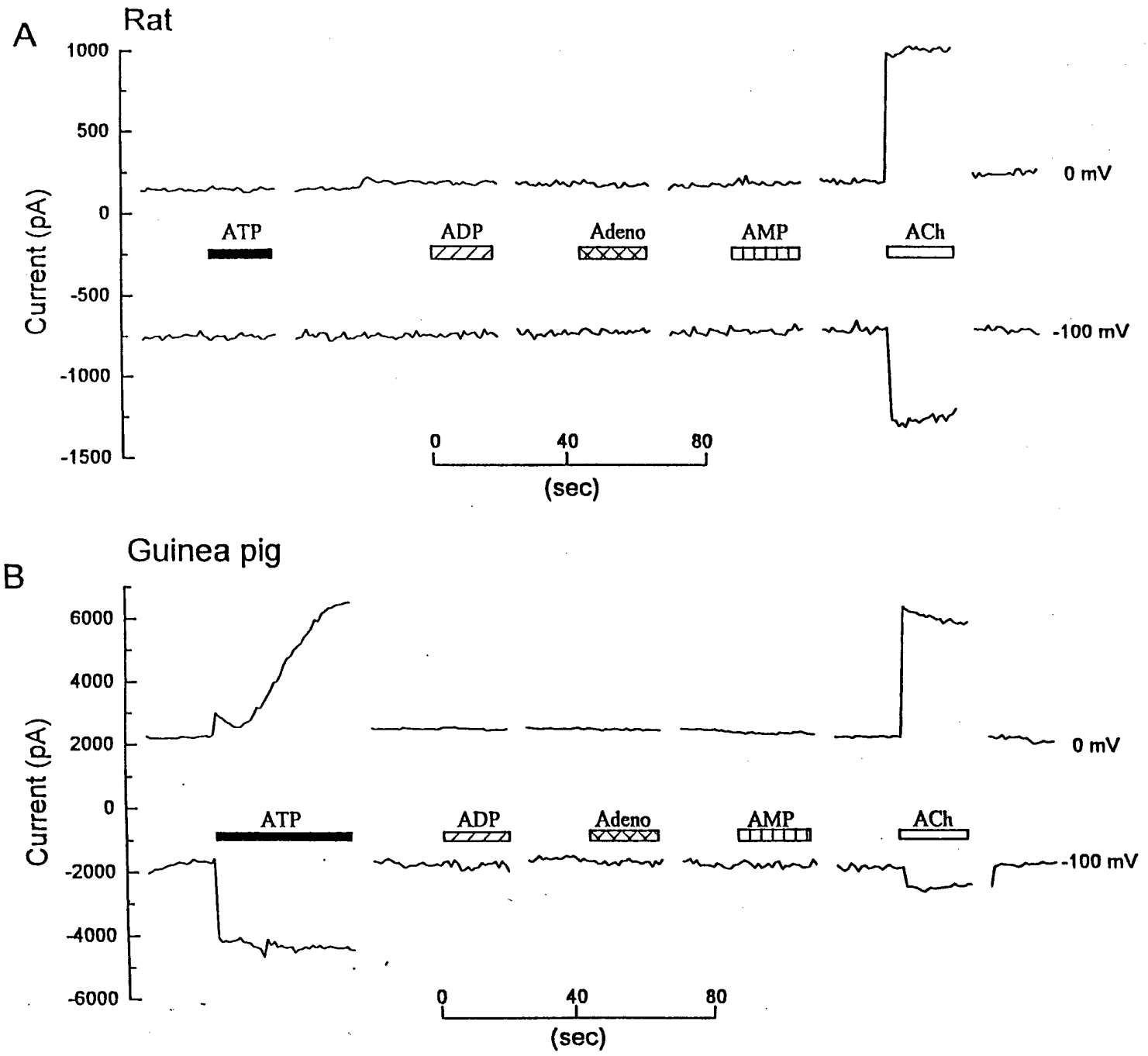
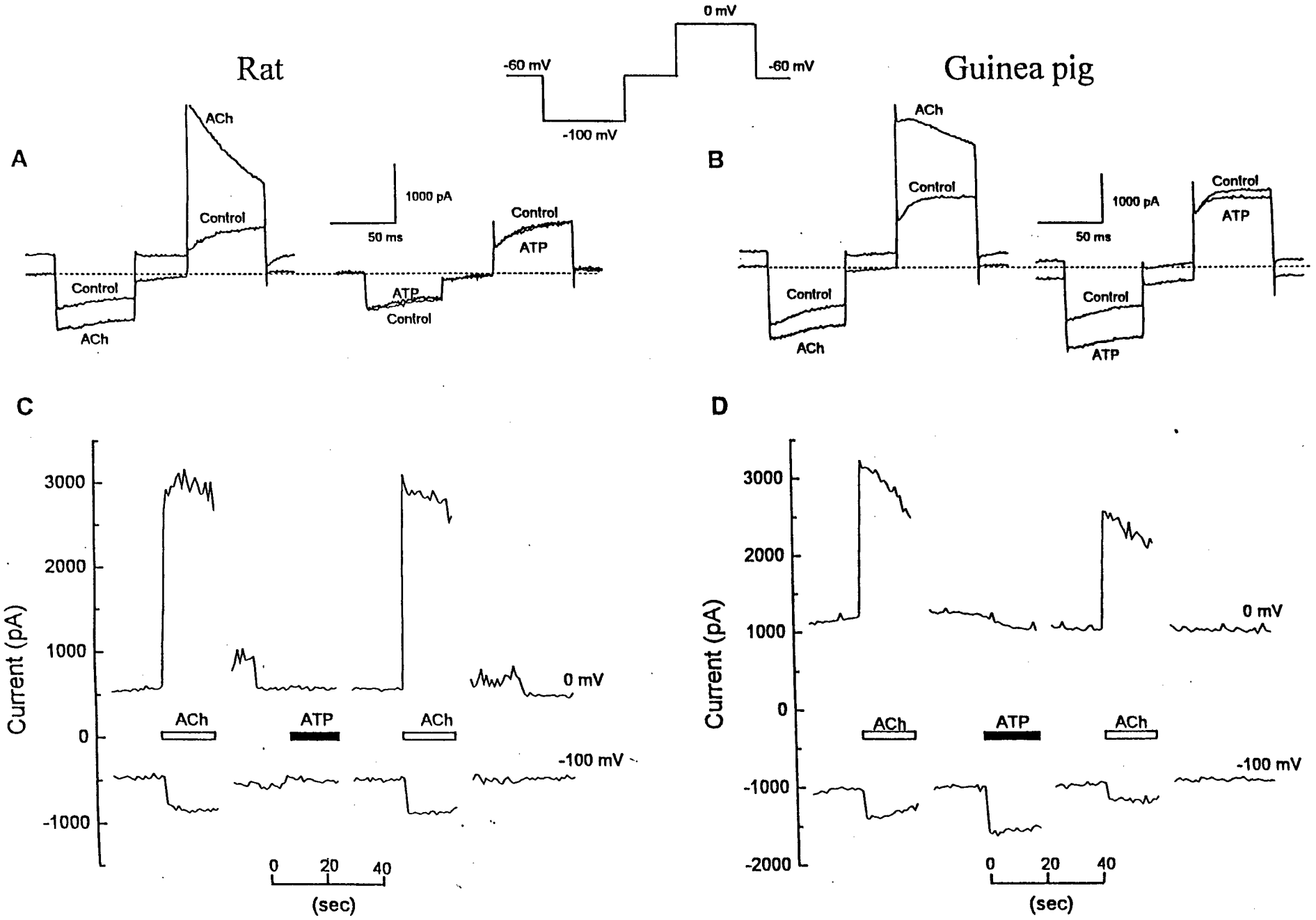
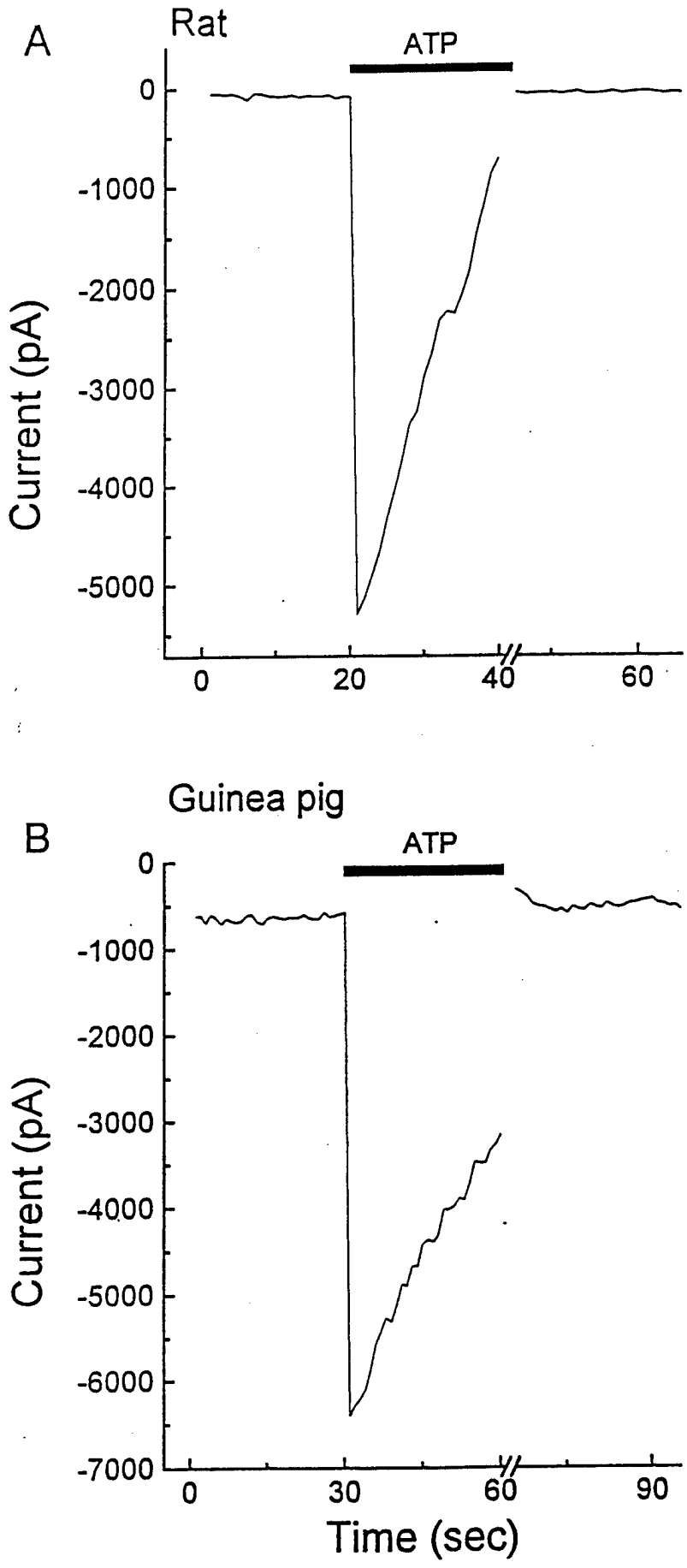
-500
-1000
-1500
-2000
-2500
-3000
-4000
-5000
0
15
30
10
20
(sec)


A
Rat
1000
500
0
-500
-1000
-1500
Current (pA)
ATP
ADP
Adeno
AMP
ACh
0 mV
-100 mV
0
40
80
(sec)
B
Guinea pig
6000
4000
2000
0
-2000
-4000
-6000
Current (pA)
ATP
ADP
Adeno
AMP
ACh
0 mV
-100 mV
0
40
80
(sec)


Rat
Guinea pig
A
B
C
D
0 mV
-60 mV
-60 mV
-100 mV
ACh
Control
ATP
1000 pA
50 ms
Current (pA)
3000
2000
1000
0
-1000
-2000
0 mV
-100 mV
0
20
40
(sec)


A
Rat
ATP
0
-1000
-2000
-3000
-4000
-5000
Current (pA)
0
20
40
60
B
Guinea pig
ATP
0
-1000
-2000
-3000
-4000
-5000
-6000
-7000
Current (pA)
0
30
60
90
Time (sec)

Comparison of Cochlear Potential vs. Click-evoked emission responses to MLS paradigm

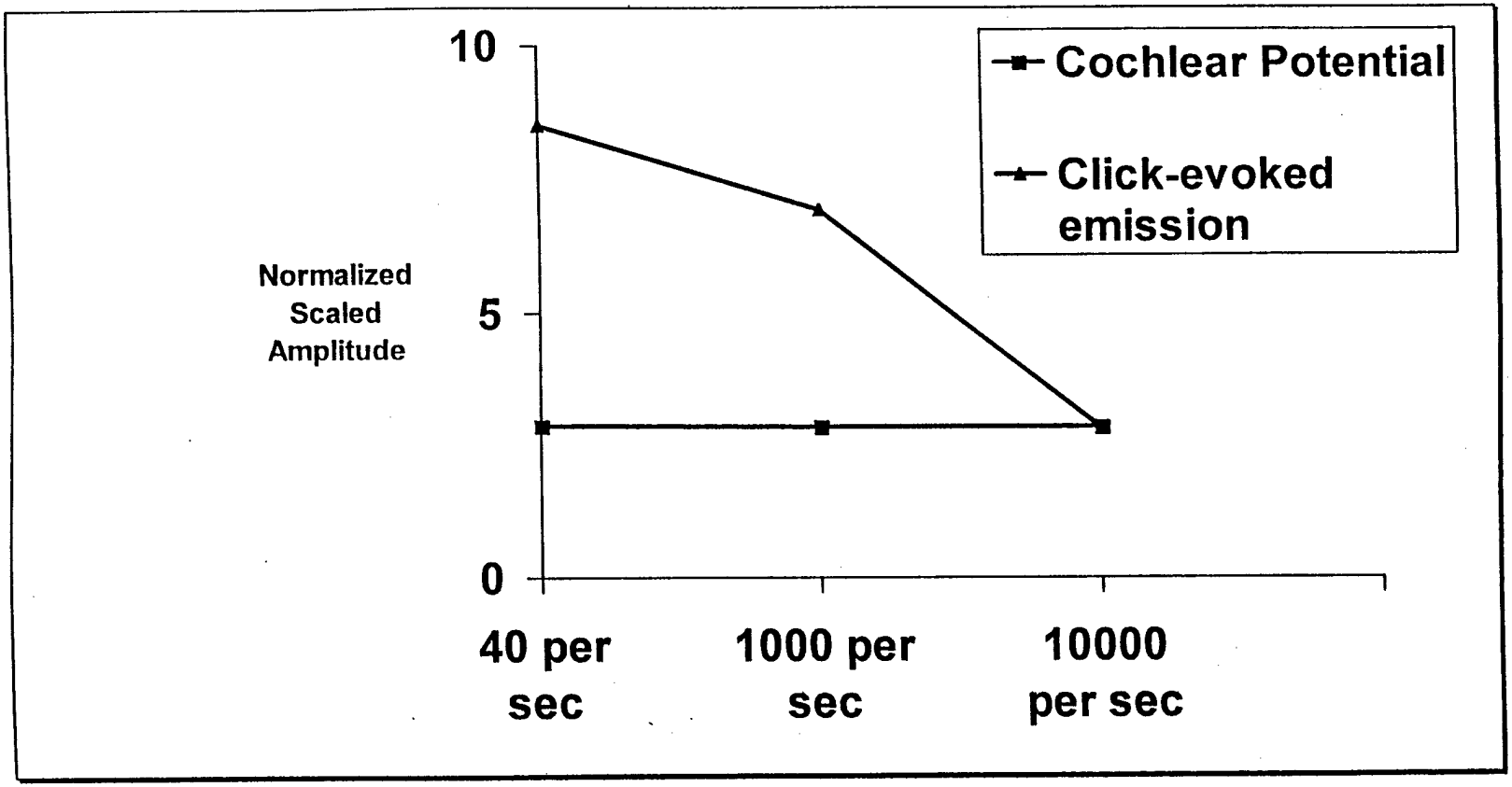


FIGURE 1

Suppression Data using 1500 Hz Tonebursts on Noise-Exposed Musicians

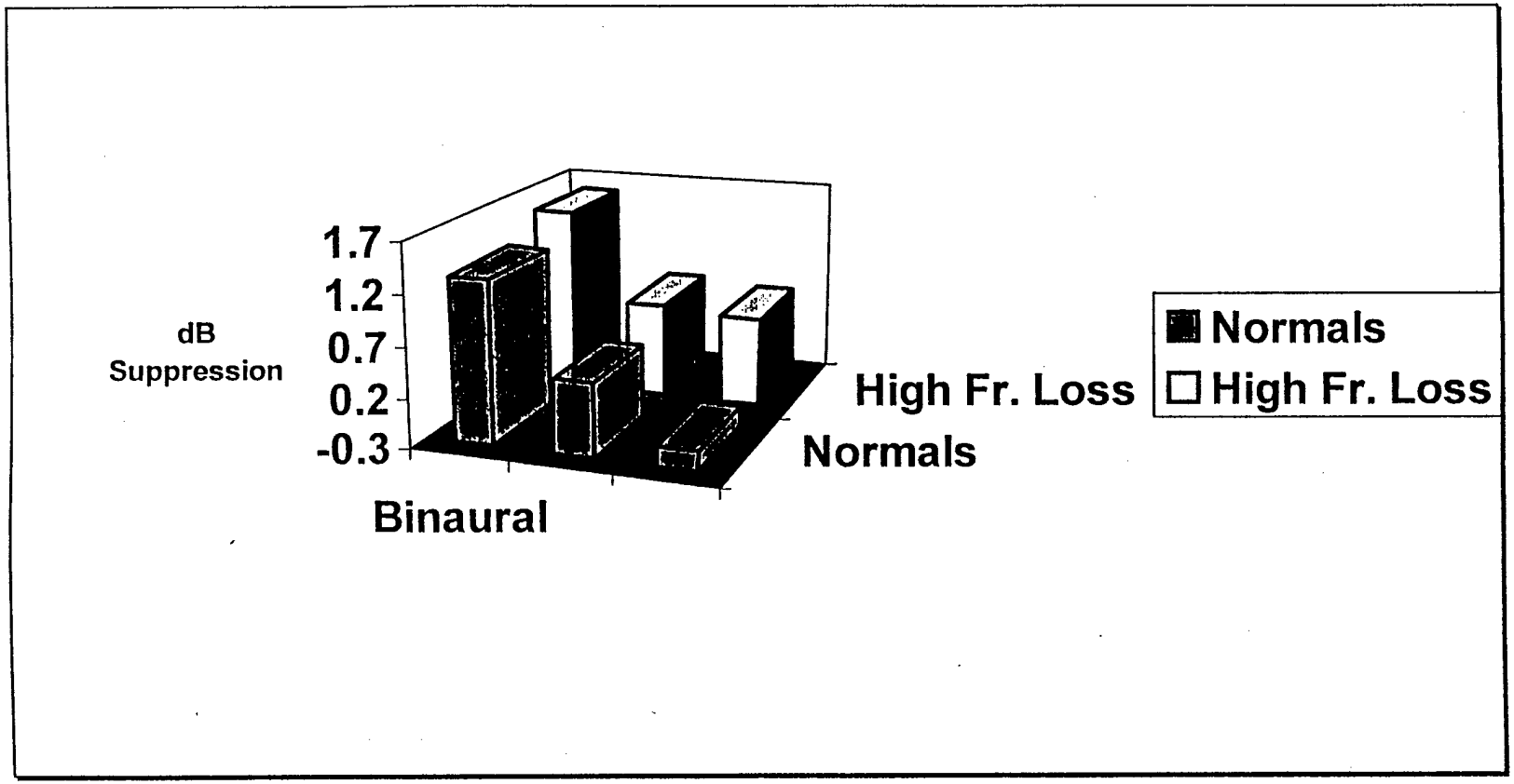


FIGURE 2

APPENDIX # 9
DAMD17-93-V-3013
Bobbin and Berlin

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HEARING RESEARCH

Binaural noise suppresses linear click-evoked otoacoustic emissions more than ipsilateral or contralateral noise

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Abstract

We studied the efferent suppression of click-evoked otoacoustic emissions with 65 dB SPL of white noise presented to left, right, or sometimes both, ears for 408 ms. Each burst of noise preceded a series of four unipolar 80 μs 65 dB peak Sound Pressure clicks, presented to the left ear only. The first click of the four-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100 or 200 ms; each subsequent click was offset by 20 additional ms via an ILO88 system with special programming modifications. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three repetitions of 600 clicks per trial. Seven subjects with normal hearing participated in the study, and three of the seven participated in a test-retest reliability study. Results showed the greatest suppression followed binaural stimulation ending within one to five ms of the first click in the pulse train. Somewhat less suppression was seen following ipsilateral stimulation. The least amount of suppression was seen following contralateral stimulation, suggesting that previous research using contralateral stimulation may underestimate efferent effects. We saw no effects when the end of the noise was 100 ms or more away from the beginning of the click train.

Keywords: Otoacoustic emissions; Efferent suppression; Binaural; Ipsilateral; Contralateral; Forward masking

1. Introduction

The medial olivocochlear system suppresses segments of outer hair cell activity when activated either contralaterally, ipsilaterally or bilaterally with an auditory stimulus of sufficient duration (Warr et al., 1986; Warr and Guinan, 1978; Puel and Rebillard, 1990; Liberman, 1989; Kujawa et al., 1993,1994).

Previous experiments on suppressing otoacoustic emissions in humans have focused mostly on the suppressive effects of continuous contralateral stimulation (e.g., Collet et al., 1990; Ryan et al., 1991; Berlin et al., 1993a,b,1995).

Kevanishvili et al. (1992) and Gobsch et al. (1992) studied forward masking of emissions in an attempt to relate the detection of hair cell suppression to perceptual masking. They used either unipolar clicks or 1000 Hz tone bursts, with time separations of 5 to 200 ms and masker durations of 6 and 50 ms; they saw little ipsilateral suppressive effect of the low level maskers on emission amplitude when judged subjectively by three independent judges.

Henson et al. (1994) offered a comprehensive summary of efferent contralateral effects on otoacoustic emissions. He noted that most of the published work showed suppression or reduction of emissions during contralateral acoustic or electrical stimulation; however, some workers, notably Brown and Norton (1990), and Plinkert and Lennarz (1992), reported an occasional increase (as well as decrease) in emission amplitude as a result of contralateral stimulation.

In this work we presented unipolar clicks to seven normal hearing human subjects to show the suppressive effects of low level binaural, ipsilateral, and contralateral white noise (408 ms in duration) in a forward masking paradigm. We used a proprietary analysis system (Wen et al., 1993) to record differences of as much as 7 dB between control and experimental traces that were not immediately apparent to casual observation.

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2. Methods

The ILO88 system is currently widely used for the recording of transient evoked emissions (Kemp et al., 1989). In the default so-called non-linear condition three 80 dB peak sound pressure pulses are presented in one polarity while a fourth pulse is presented out of phase with the first three and at 10 dB greater intensity. The resulting echo represents the difference between the sum of the three echoes elicited at 80 dB and the one out-of-phase echo elicited by the 90 dB pulse; the strategy is designed to cancel any artifactual ringing which could be mistaken for hair cell echoes.

Suppression is evident, but not robust, with these default conditions (Berlin et al., 1993b). In contrast, efferent effects are more readily seen at low intensities than at high and with linear rather than non-linear click trains (Collet et al., 1990; Hood et al., 1994); therefore, we designed our experiments around low level linear clicks and noises.

One of us (D.K.) supplied the program for an ILO88 system to control the temporal interval between the offset of a duration-controlled noise stimulus and the onset of a 4-click train. We selected a 408 ms duration noise based on the work of Liberman (1989) and Huang et al. (1994) to maximize the likelihood of activating the efferent system. The emission-evoking 80 μs clicks were all 65 dB peak sound pressure and all of the same polarity. The first click of the 4-click group followed the end of the noise by either 1, 2, 5, 10, 20, 50, 100, or 200 ms, whereas the final 3 clicks followed the first click by successive increments of 20 ms. Conditions were alternated so that a 'without noise' condition preceded a 'with noise' condition for three complete trials. The order of ipsilateral vs. contralateral vs. bilateral presentation of the noise was counterbalanced among subjects for the 24 different listening conditions. Three subjects were retested under conditions where the first of the 4 clicks began 1 ms after the termination of the noise and each subsequent click was offset by 20 ms.

Each set of control vs. experimental data consisted of the mean of three 'without noise' trials, and three 'with noise' trials, compared to one another using two separate quantification systems. In one case we compared the mean echoes to each other using the Kemp aggregate echo level number, which we called 'dB-ILO'. This number represents the overall spectral amplitude of the echoes averaged by the ILO88 system over a 20.48 ms window. The second method used the Kresge Echomaster system (Wen et al., 1993) which allows custom designed amplitude, time and frequency comparisons between means of control and experimental conditions. We chose to quantify the RMS differences between the echoes in two ms segments and labeled this number 'dB-K' to differentiate it from the overall RMS number available from the ILO88 system. The Kresge Echomaster system also allowed us to make temporal comparison of differences between segments of the control and experimental echoes in 40 μs steps (See Table 1 later for examples).

3. Results

The binaural noise condition generated 1.5 to 2 dB-ILO of emission suppression when the noise preceded the first click in the train by one to twenty ms. Thereafter the suppressive effects decreased as time-separation increased.

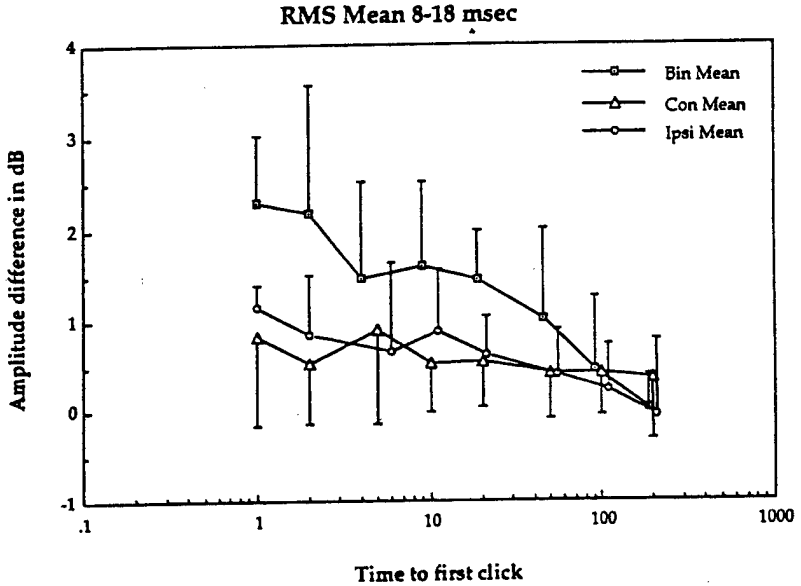

Fig. 1. The effects of 408 msecs of binaural, contralateral, or ipsilateral noise preceding a left ear click train in which the first click started at 1, 2, 5, 10, 20, 50, 100, and 200 msecs after the end of the noise burst. Each of the subsequent clicks was offset by an additional 20 msecs. The mean and 1 Standard Deviation are presented using the amplitude difference data between 8–18 msecs from the Kresge Echomaster (KEM) 3.0 program.

The amount of suppression to binaural noise shown in Fig. 1 is 2.5 to 4 dB-K between 8 and 18 ms after the click stimulation, a zone in which the majority of suppression is seen. The values for ipsilateral and contralateral suppression are respectively smaller in this forward masking paradigm. Fig. 2 compares suppressive effects of binaural, ipsilateral and contralateral noise at 1, 10, 50 and 200 ms time separations between the end of the noise and start of the first click. The suppression is expressed in dB-K.

Fig. 3 offers another view of the same data. Here we compare the data in dB-K over the 4 to 20 ms period of the KEM 3.1 program analysis, showing the binaural data only when the noise terminates 1, 5, 20, and 200 ms before the first click. Then in the companion figure we show similar binaural data for time-separations of 2, 10, 50 and 100 ms between the end of the noise and the beginning of the first click. Again three primary trends are evident:

1. The shorter the time-separation between the end of the noise and first click, the greater the suppressive effect ($F = 6.44$; $df = 7,42$; $P < 0.001$). Duncan's Range Test showed that 1 ms time separation is most effective. There were no significant differences between 2, 5, 10, and 20 ms of separation. Thereafter separations of 50, 100, and 200 ms all differed significantly from one another.
2. Suppression of 2.5 to 3.5 dB-K takes place in the 8–18 ms zone after click onset ($F = 10.37$; $df = 8,48$; $P < 0.001$). Duncan's range Test showed that the greatest effects occur between 18 and 20 ms. No significant differences were seen between 10 and 18 ms, while the least suppression was seen in the 2–8 ms ranges.
3. Binaural noise generates more efferent suppression than either ipsilateral or contralateral noise ($F = 11.43$; $df = 2,12$; $P < 0.005$). Duncan's Range Test showed that, while binaural noise generated the most suppression, ipsilateral and contralateral stimulation did not differ from one another in their suppressive abilities, although they did exert significant amounts of suppression. We also observed that the sum of ipsilateral and contralateral suppression was within 0.8 of a dB of the suppression generated by binaural noise as was predicted by Kirk and Johnstone (1993).

Spectral analysis through a Hanning window available in the KEM 3.1 program showed a gradual shift in the largest spectral difference (at 2344 Hz) from 6.328 dB of binaural-noise-induced-suppression when the first click

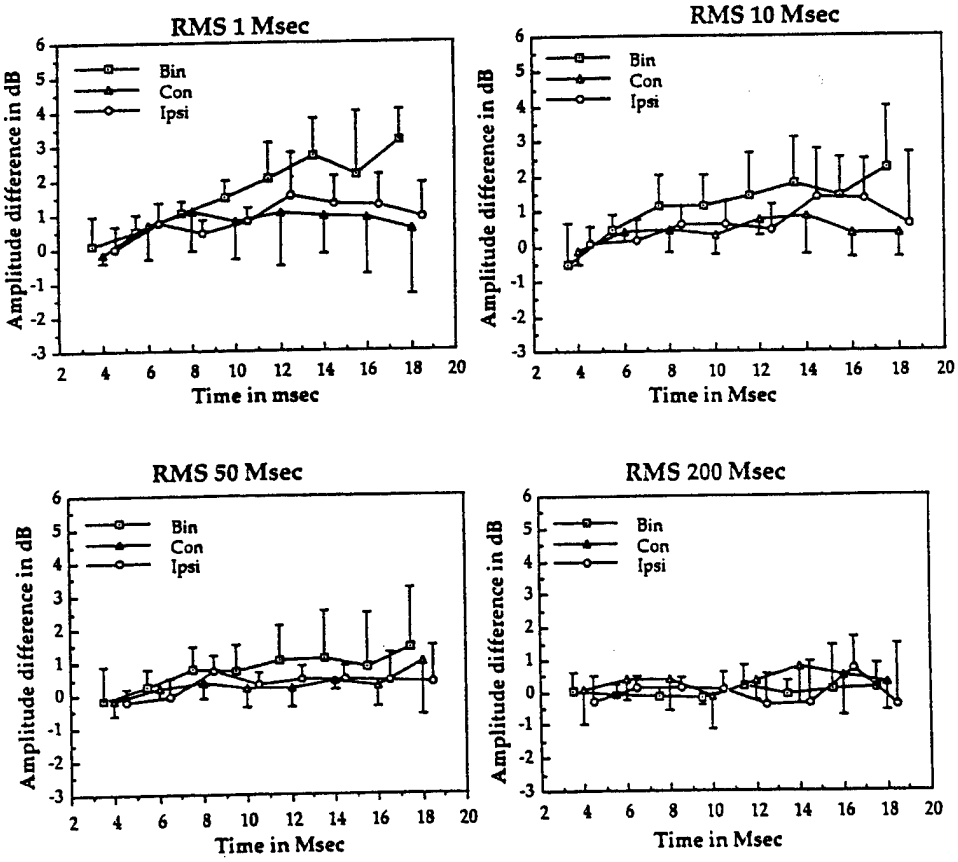

Fig. 2. The emission-suppressing effects of binaural, ipsilateral and contralateral noise. Data, expressed in db-K, are grouped by time intervals in which the noise preceded the first of four click-evoked emission by either 1, 10, 50 or 200 msecs.

followed the noise by only one ms, dropping to 2.267 dB at 100 ms time separation, and then down to 0.394 dB at 200 ms time separation for one representative subject (C.B.). Other subjects showed qualitatively similar suppression in spectral zones between 1000 and 3000 Hz.

We checked test-retest reliability for three subjects. Data for one subject are shown in Fig. 4. Group analysis shows reliability as high as 0.62 for the binaural data at 1 ms time-separation but only 0.13 for the ipsilateral data and 0.046 for the contralateral data. Reliability in general fell as the time separation between the end of the noise and the onset of the click increased. This observation suggests that the binaural effects are clearly more robust and more reliable than either ipsilateral or contralateral stimulation in this forward masking paradigm with humans.

4. Discussion

There are two major efferent systems which can affect the emissions reaching the recording microphone: the middle ear muscle reflex efferent motor loop, and the olivocochlear efferents. The 80 μs click stimuli in this experiment were just detectable by our normal subjects at 38 dB peak sound pressure. Thus, since the clicks used in the experiment were presented at 65 dB peak sound pressure, they were only at 27 dB HL, a level far too faint to evoke a clinically measurable middle ear muscle reflex. The threshold for the noise was 22 dB SPL; therefore, the 65 dB SPL noise was only 43 dB HL, also a level far too low to elicit a clinical middle ear muscle reflex.

The suppressive effects are actually largest when the clicks and the noise are at lower intensities. We know this from separate work completed after this present experiment had started, which showed us that the suppressive effects of contralateral noise were largest when the clicks were at 55 dB peak sound pressure; the relative effects diminished when either the noise or the clicks were presented at higher intensities (Hood et al., 1994), a phenomenon also reported by Collet et al. (1990).

Finally, studies of people with no middle ear muscle function (e.g., Collet et al., 1990; Berlin et al., 1993a) all suggest that the middle ear muscle reflex did not partici-

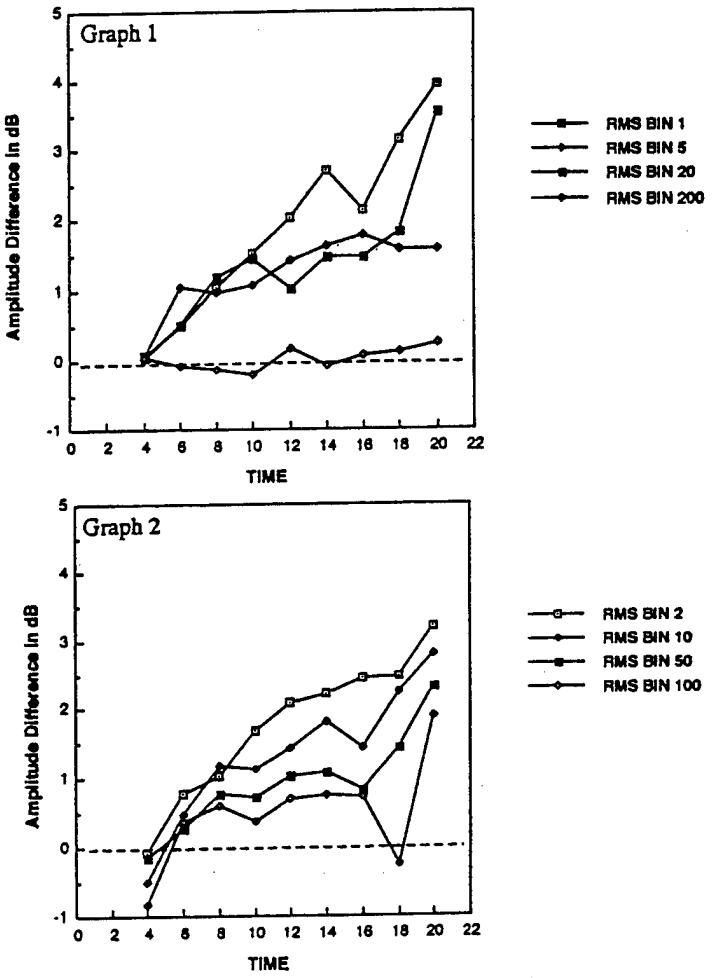

Fig. 3. Shows binaural noise suppression data for 1, 5, 20 and 200 msecs of time-separation between the end of the noise and the beginning of the first of four clicks. The companion figure shows the data for 2, 10, 50 and 100 msecs. Data are expressed in db-K.

pate in this experiment, although of course it is impossible to completely rule out subclinical middle ear muscle contraction in any one subject.

Still another potential confounding problem in such experiments is acoustic crosstalk. If standard ear phones such as TDH-39 with MX 41-AR cushions had been used, crossover by bone conduction or even partly by air conduction could conceivably take place at levels as low as 40 dB HL. However, in this experiment we used insert earpieces from the Kemp system; the psychophysical crossover in similar insert earphones exceeds 70–90 dB in frequencies below 1000 Hz and 60–70 dB in frequencies above 1000 Hz (Killion, 1984).

Liberman and Brown (1986) showed almost no efferent suppression in cats stimulated by 25 ms or less of noise stimulation; optimum durations to activate the efferents were reported to reach an asymptote between 50 and 500 ms. Within the limits of the technical difficulties to be described, our findings suggest that the human efferent system overlaps at least one part of the time-frame seen in cats.

Binaural stimulation in the forward masking paradigm predictably elicited more robust and more reliable efferent suppression of evoked otoacoustic emissions than either ipsilateral or contralateral stimulation. In absolute numerical terms, however, more suppression is seen with continuous 60 dB SPL contralateral noise stimulation when the click is at 55 dB peak Sound Pressure (≈ 17 dB HL) than we see in the binaural condition in this forward masking experiment (Hood et al., 1994; Berlin et al., 1995). This observation is to be expected because of the forward masking nature of the paradigm; the continuously running noise paradigm would confound data collection during conditions of ipsilateral and binaural efferent stimulation.

5. Technical difficulties

We recognize several problems with the data presentation in this experiment. Because of constraints in the available software, the click stimuli could only be deliv-

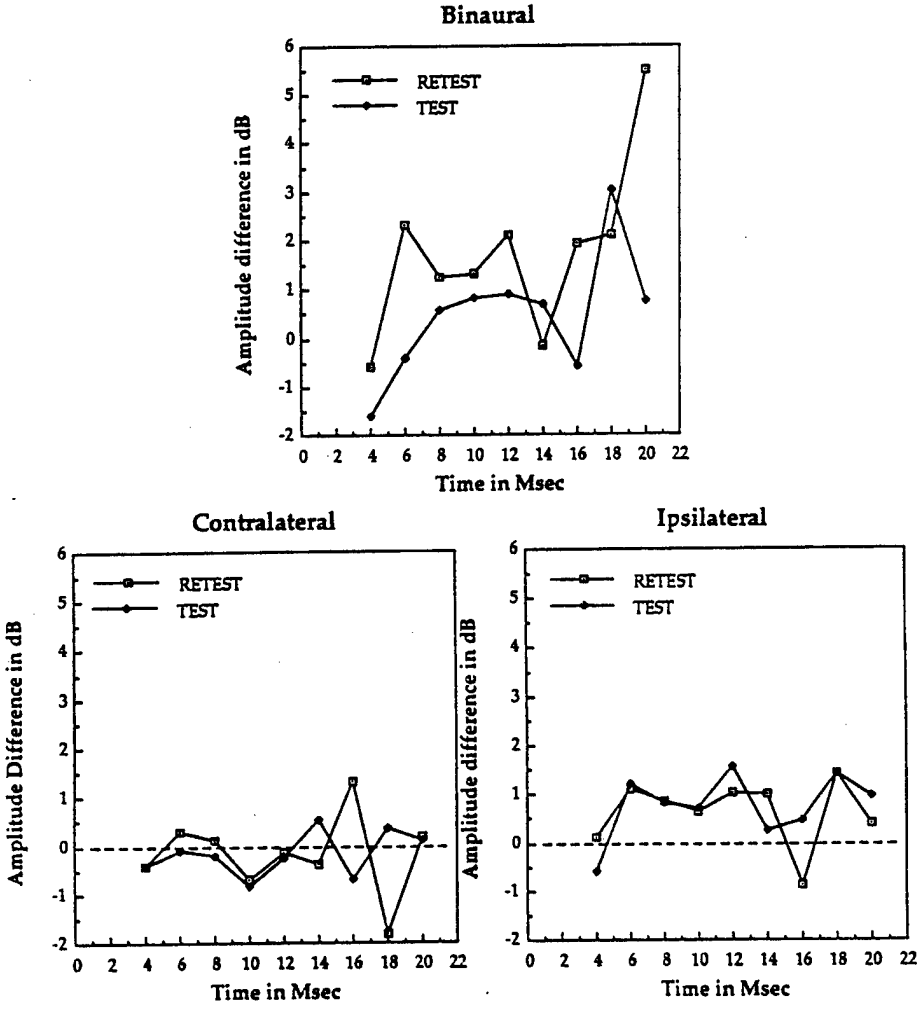

Fig. 4. One subject's test-retest reliability for the time separation of 1 ms between the end of the binaural, ipsilateral or contralateral noise.

ered in packets of four stimuli per stimulation unit. Thus, when we described a click train as beginning '1 ms after the end of the noise,' it was only the first of the 4 clicks that was one ms away from the end of the noise. The other three pulses were 21, 41, and 61 ms away from the end of the noise respectively. Yet each of the responses to the clicks is added into the average obtained by the ILO88. Similarly, in the 50 ms condition the last 3 clicks were presented 71, 91, and 111 ms after the end of the noise. Thus, whatever efferent effects we report here are likely to have been attenuated because three-quarters of the 600 clicks used to comprise a single file were 20–60 ms later than the intended time-relationship to the end of the noise.

We are indebted to G.I. Frolenkov (Frolenkov et al., 1995 and Tavartkiladze et al., 1995) for pointing out to us that, in order to avoid acoustic interaction between the end of the noise and the beginning of the first click, we should have allowed the noise about 5 ms to decay. This was not a fatal flaw in any of our data collection since only the first click in the series was within 1–5 ms of the offset of the noise; all subsequent clicks were offset by at least 20 ms.

If we were to present only a single click after the end of the noise, the 80 ms window averaging paradigm used by the ILO88 would still include the data from the three subsequent empty 'vacated' bins as 'noise' and would attenuate the apparent size of the averaged evoked emissions by a factor of three. [We have recently observed that a 1-click experiment in this paradigm yields about the same data as a 4-click train experiment; in addition we find that there may be gender differences, laterality effects and occasionally even reverse suppression effects which have to be taken into consideration in future experiments (Berlin et al., 1995; Barham et al., 1995)].

The broad-band click and broad-band noises potentially ignore frequency specificity reported in experiments of this sort (e.g., Liberman, 1989). Thus an improved experiment would have all of the echo-evoking stimuli in the same time registration with respect to the end of the noise, would include the echoes from only a single 20.48 ms bin following the click, would take into consideration ear and gender effects, and would focus on various frequency bands, where presumably the effects might be even larger than we report here.

6. Of what value is a 3 to 6 dB effect in the auditory system?

A forward masking effect of 3–6 dB-K suppression of hair cell activity, which we saw when both ears were exposed to approximately a half-second of noise, would be even larger if it could be measured while the noise were continuously active. This work supports Liberman's prediction that 200 ms or more durations of noise would adapt outer hair cell function leading to a change in the excitation pattern of inner hair cells and single units. Liberman proposed that the presence of the noise probably changed the baseline operating characteristics of the outer hair cells

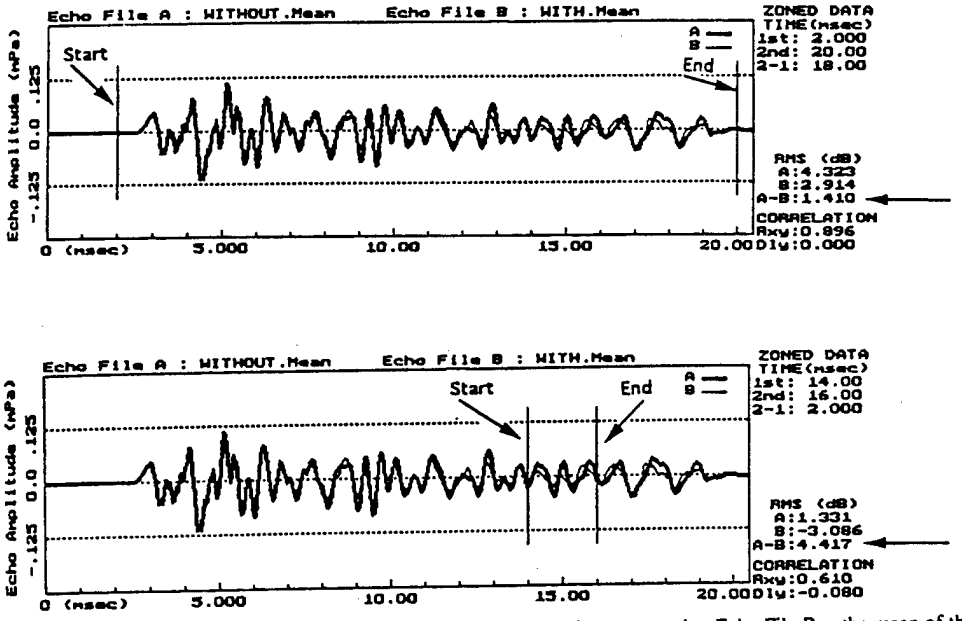

Fig. 5. Two averaged emissions traces (Echo File A = the mean of three trials without noise compared to Echo File B = the mean of three trials with noise) appear to have only 1.410 dB difference between them when scanned from 2 through 20 msecs (vertical bars marked **Start** and **End**) but, in fact, have 4.417 dB amplitude differences between them when scanned between 14 through 16 msecs after stimulation. See numerical codes to the right of each display for clarification. Companion Table 1 shows actual data values for these traces for Time Segment in msecs, Rxy (correlation), RMS differences, and Time Delay between traces in msecs.

and would adapt single units faster, in preparation for upcoming transients; if such a shift were to be applied to the sharply rising edge of a speech intelligibility curve such as the articulation index, (Pavlovic, 1994) it could conceivably improve connected speech intelligibility in a borderline noisy situation by as much as 40–60% (see for example Humes et al., 1986; Pavlovic, 1994; Hood et al., 1991). Thus, shifts in the baseline from which listening in noise takes place, or anti-maski9X phenomena as outlined by Hirsh (1948), Licklider (1948), Nieder and Nieder (1970), Kawase et al. (1993), or Kawase and Liberman (1993), might be traced to outer hair cell changes controlled by the efferent nervous system, which helps to facilitate listening in noise. We would not expect the effects of efferent function to be dramatic and outstanding in humans (cf., Scharf et al., 1994) without the conditions which mimic real-life listening conditions, including (preferably) binaural presence of long durations ($\geq$ 200 ms) of noise. However, Henson et al. (1994) suggest that cochlear reverberation may be reduced through activation of the efferents in bats. A similar reduction in cochlear reverberation, as yet unreported in humans, would serve to essentially improve the signal-to-noise ratio whenever it occurred.

7. Relationship to other forward masking work on emissions

Kevanishvili et al. (1992) and Gobsch et al. (1992) presented work studying the relationship of forward masking to emission suppression. They studied whether perceptual masking occurred at the hair cell and cochlear partition level, or at more central levels. They assumed that if masking were taking place at the pre-neural level, perceptual masking and masking of the emissions would occur at the same intensities and durations. They reported that the perceptual masking functions and masking of the emissions were quite separate, and in fact used maskers of 80 and 68 dB SPL to prove that point. Whether or not they induced middle ear muscle reflexes is debatable, but the 5 ms delay between noise offset and click onset should have vitiated most if not all of those effects. Yet these conscientious workers did not report that they saw much masking of the emissions. Our contention, after looking at their waveforms, is that there are many amplitude and phase changes in the tracings that they published, which could be easily ignored by someone looking simply at patterns but that could be quantified by summing traces from similar conditions and overlapping conditions with and without masking. Any deviations from perfect correlation might be due to either noise or phase and amplitude changes. Our analysis program is capable of quantifying such a change, where the eye is not. For example, using data that do not appear to show much difference between traces by casual inspection, we can show differences of 4.417 dB (Fig. 5) between 14 and 16 ms but only 1.4 dB when viewed globally over a 2–20 ms period after stimulation. Such minuscule visual differences in Gobsch et al. (1992) and Kevanishvili et al. (1992), could easily be overlooked by naked eye scanning unless one overlapped the traces and made a systematic point-by-point analysis. The numbers from such an analysis, taken in 2 ms steps, is provided in Table 1. The first column lists the selected time segments. The second column shows the correlation between the 'without' and 'with' noise traces in each time segment, the third column the RMS amplitude differences in dB in each time segment, and the fourth column shows any shifts in ms between the two traces. The resolution here is 40 μs per point.

Table 1
Echo R_{XY}, RMS differences and delay values for Fig. 5

Time (ms)	R_{xy} (x = A, y = B)	RMS (A–B in dB)	Delay (B to A in ms)
2.00– 4.00	0.972	0.14	0.000
4.00– 6.00	0.964	0.35	0.000
6.00– 8.00	0.950	0.99	0.000
8.00–10.00	0.867	1.96	−0.040
10.00–12.00	0.913	2.36	−0.040
12.00–14.00	0.836	3.21	−0.040
14.00–16.00	0.610	4.42	−0.080
16.00–18.00	0.771	2.48	−0.080
18.00–20.00	0.809	3.72	−0.040

One other difference must be addressed. Gobsch and Kevanishvili first collected emissions data without masking. Then they conducted their masking experiments and, at the end of the masking experiments, collected emissions again to clicks alone. They did not report any changes between the control conditions before and after the experiment. We have found that unless we alternated the conditions of testing (three conditions each of 'without' alternated with three conditions of 'with' masking) we would see a small but measurable change in the baseline of emission amplitude without any contralateral stimulation, which could have obscured our visualization of experimentally induced suppression.

8. Conclusions

We studied the suppressive effects of binaural, contralateral and ipsilateral white noise on linear TEOAEs. Binaural stimulation elicits the most suppression of otoacoustic emissions in a forward masking paradigm when the onset of the click train is 20 ms or less after the offset of a 408 ms white noise burst. Less suppression occurred to ipsilateral or contralateral stimulation, and the suppression essentially disappeared when the end of the noise was 100 ms or more away from the beginning of the click train.

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6

Hearing Aids: Only for Hearing-Impaired Patients with Abnormal Otoacoustic Emissions

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The purpose of this chapter is to show that many patients have carried a misdiagnosis of "nerve damage" for years, a diagnosis that has semantically discouraged them from using hearing aids successfully. In fact, we can now discriminate between hair cell loss and primary neural disease and can consider using specialized types of dynamic compression hearing aids for patients with confirmed and isolated outer hair cell dysfunction. In contrast, patients with primary auditory neuropathies are *not* hearing aid candidates.

HOW DOES ONE DISCRIMINATE BETWEEN HAIR CELL AND NEURAL HEARING LOSS?

Outer Hair Cell Loss

Patients who have outer hair cell loss have audiograms that fall between 20 and 75 dB. Their otoacoustic emissions are absent, yet they have robust and synchronous neural discharges to clicks (Figure 6–1). This latter find-

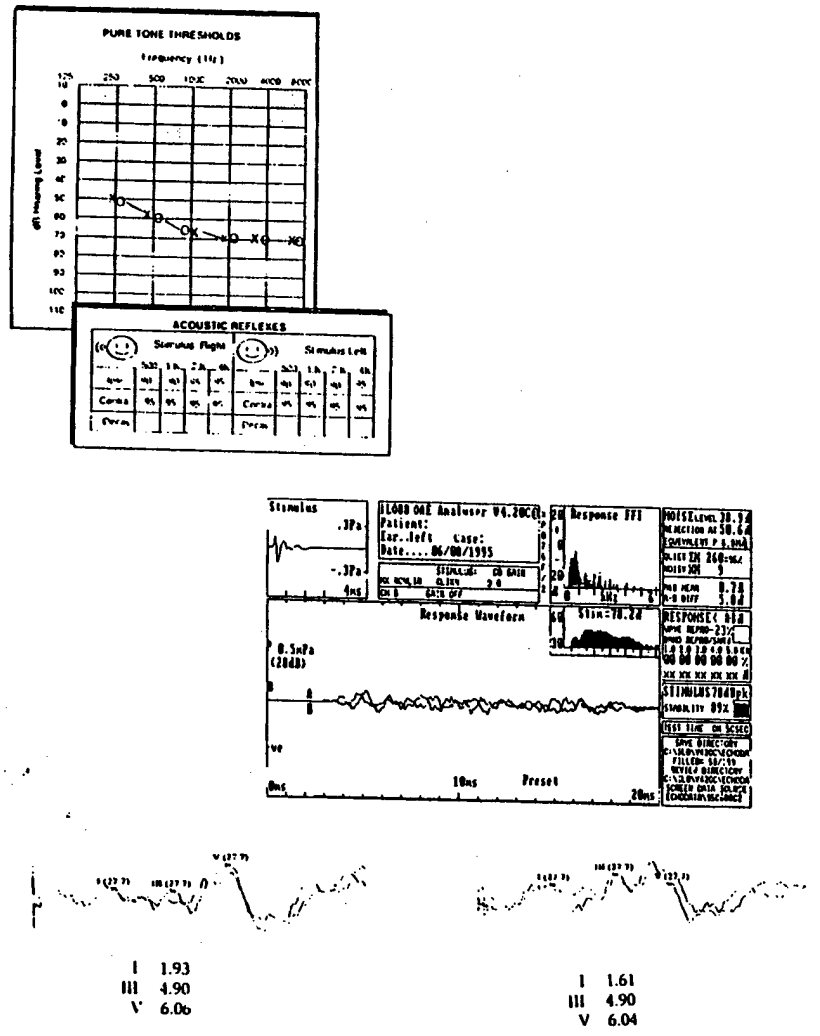


Figure 6–1. Audiogram emissions and ABR from a person with a fittable cochlear hearing loss.

ing is indicated by well-formed ABR responses with good morphology and normal absolute and interpeak latencies to high intensity, 85- to 95 dB HLn, stimuli. These are good candidates for hearing aids, and are likely to have good results, providing there are no inflammatory or autoimmune processes which disrupt speech coding.

Inner Hair Cell and Spiral Ganglion Loss

Patients who have no emissions and no ABR to air-conducted clicks ar likely to have suffered some inner hair cell and spiral ganglion loss. I they are being considered for cochlear implants, electric ABRs shoul reveal synchronous neural discharge if properly administered and inter preted, thus, with somewhat circular reasoning, confirming their candi dacy for cochlear implantation. However, many patients are successfu implant candidates despite poor electrical ABR tests.

Primary Neuropathies

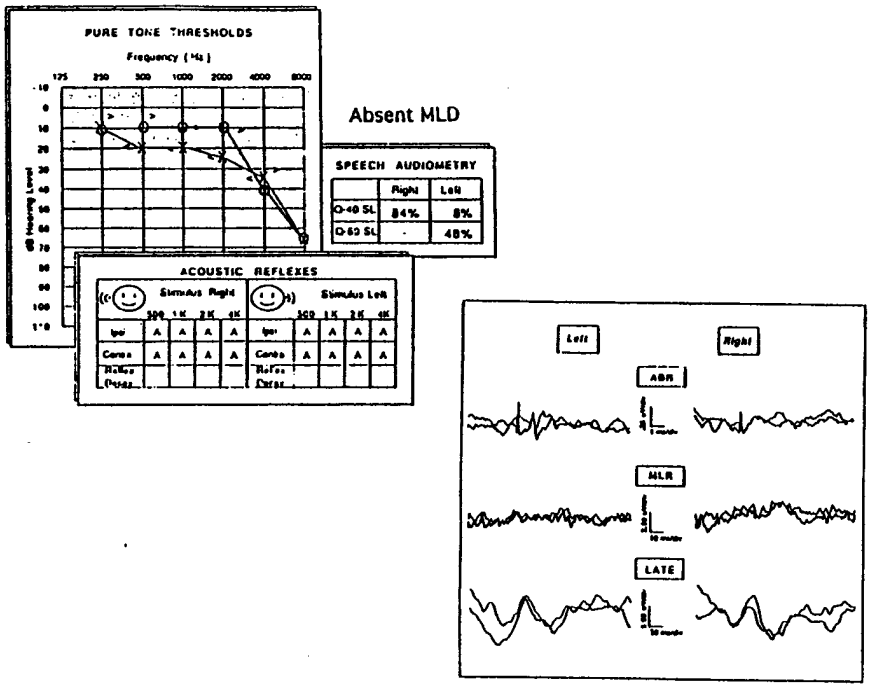
Finally, there is a small group of patients who have no ABR but have nor mal hair cell emissions. Some of these patients show little or no hearin loss by pure tone audiometry (Figure 6–2), others show very poor audio grams (Figure 6–3), still others have thresholds that fall somewhere i between (Figure 6–4). All show: (a) absent middle ear muscle reflexes, (b absent MLDs, (c) very large otoacoustic emissions, and (d) virtually nc efferent effects of contralateral ipsilateral or binaural noise on thei robust click-evoked otoacoustic emissions (see sample in Figure 6–5 anc explanation later; Berlin et al. 1994; Sininger, Hood, Starr, Berlin & Picton 1995; Starr et al., 1991). Hood, Berlin, Hurley, and Wen have shown how we quantify efferent suppression in Chapter 3.

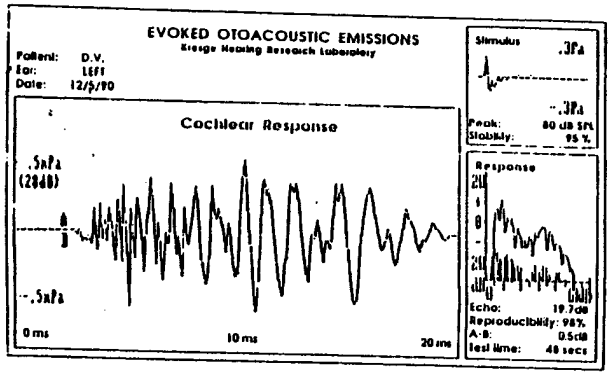
These patients probably have some form of primary auditory neuropa thy which makes them poor candidates for hearing aids (Berlin et al., 1993 1994) . Related evidence suggests that these patients also have other form of nonauditory primary neuropathy like Charcot-Marie-Tooth syndrome o some similar disease that desynchronizes single unit responses in othe motor as well as sensory units (Berlin et al., 1994; Sininger et al., 1995).

SUPPRESSION OF OTOACOUSTIC EMISSIONS

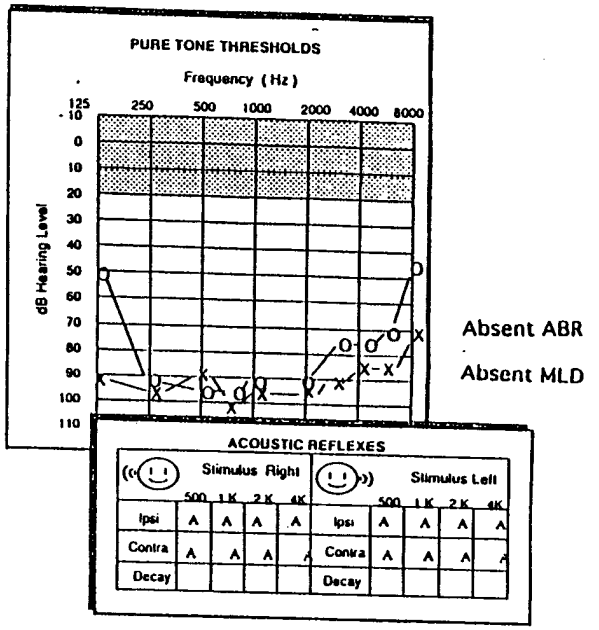
The medial olivocochlear system suppresses segments of outer hair cel activity when activated either contralaterally, ipsilaterally, or bilaterall with an auditory stimulus of sufficient duration (Kujawa et al., 1991 Liberman, 1989; Puel & Rebillard, 1990; Warr & Guinan, 1978; Wari Guinan, & White, 1986).

In the previous chapter, Hood outlined our techniques for recordin otoacoustic emissions and quantifying the efferent suppression that is th hallmark of the integrity of the afferent-efferent loop. In normal hearin subjects the suppression can approach 2 to 4 dB K the [illegible]





Figures 6–2 to 6–4. Audiograms and reflex are typical of those found in some patients with auditory neuropathy. Patients all have normal otoacoustic emissions and absent ABRs, MLDs, middle ear muscle reflexes, and poor speech discrimination in quiet as well as in noise. The patients universally showed no contralateral suppression when assessed by the Wen Kresge Echomaster Program. The kernel point is that the pure tone audiogram alone *cannot* be used to predict hearing aid need, success, or use, unless it is combined at least once with an otoacoustic emissions test showing absent or reduced emissions. *(continued)*



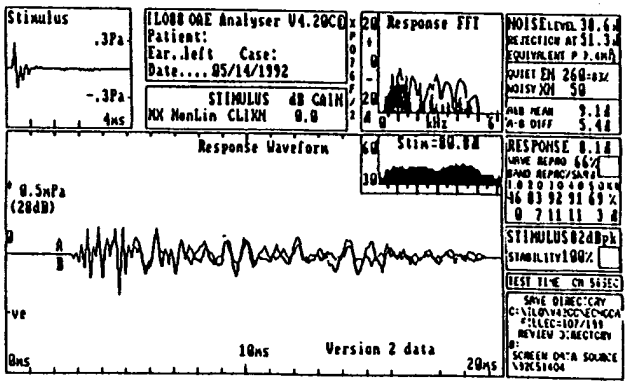


Figure 6–3. See Figure 6–2.

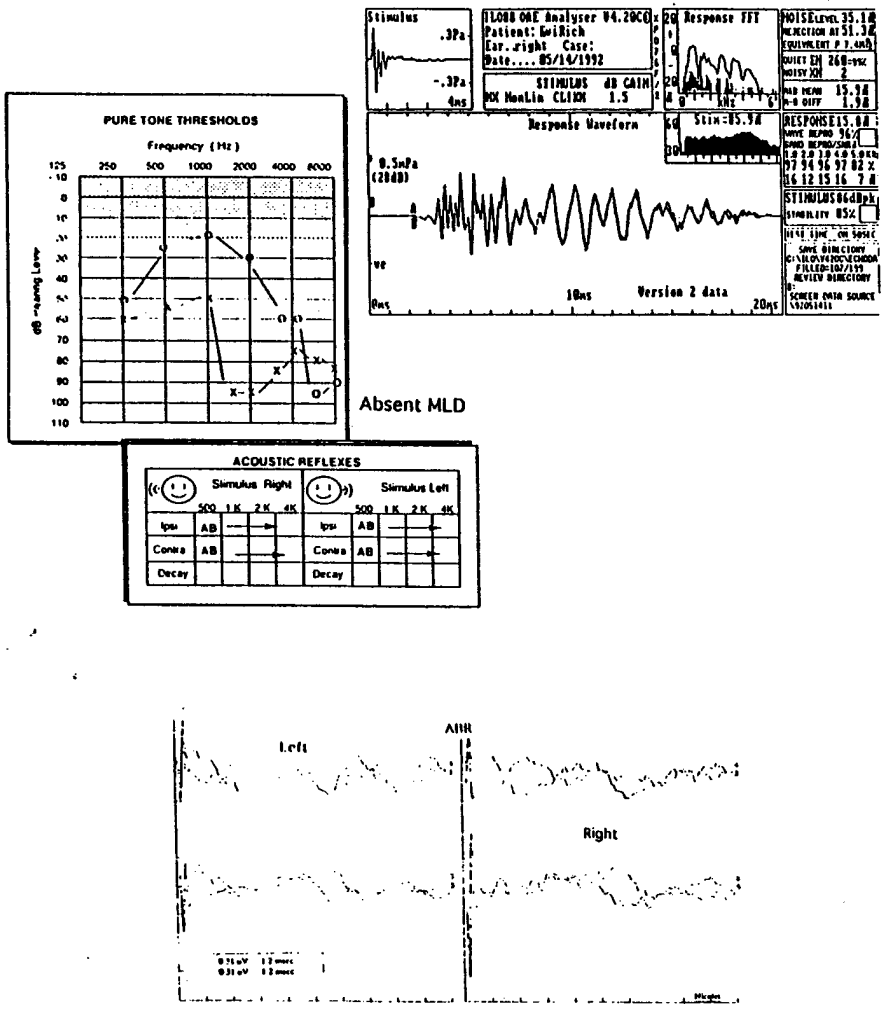
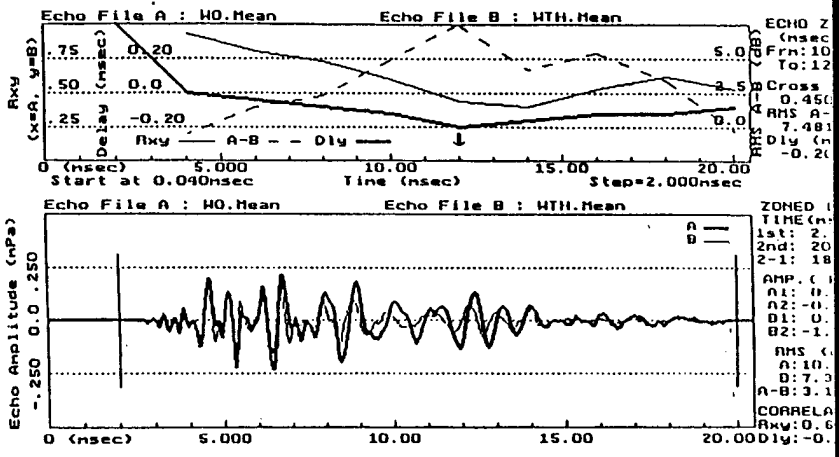
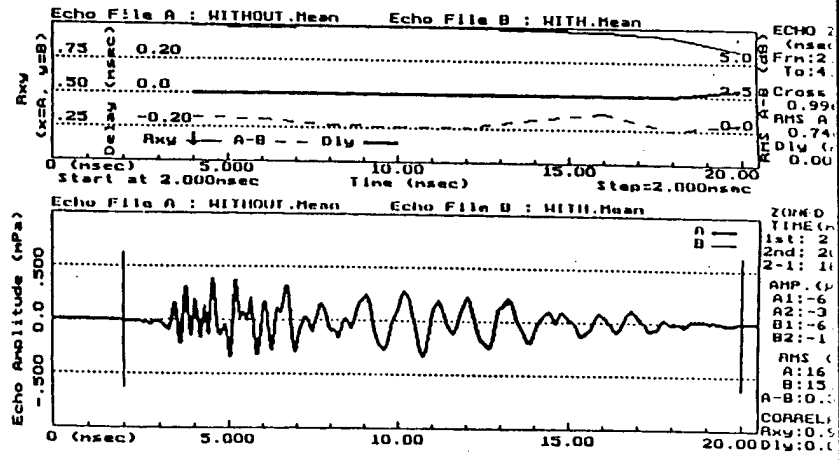


Figure 6–4. See Figure 6–2.



A



B

Figure 6–5. A: A typical click-evoked emission obtained from subjects with no hearing. B: Three virtually parallel lines depicting the absence of suppression i of the patients with auditory neuropathy.

for the measurements completed with Han Wen's Kresge Echomaster System (1993; Figure 6–5a). Figure 6–5b comes from a patient with auditory neuropathy and shows the pathologic three parallel lines common to all of these patients when studied by efferent suppression techniques. Clearly the nature of their pure tone audiograms is misleading with respect to hearing aid need or success.

Patient 1

This patient was sent to us as part of our search for Ultra-audiometric subjects (Berlin et al., 1978). Her rising audiogram (Figure 6–6) suggested

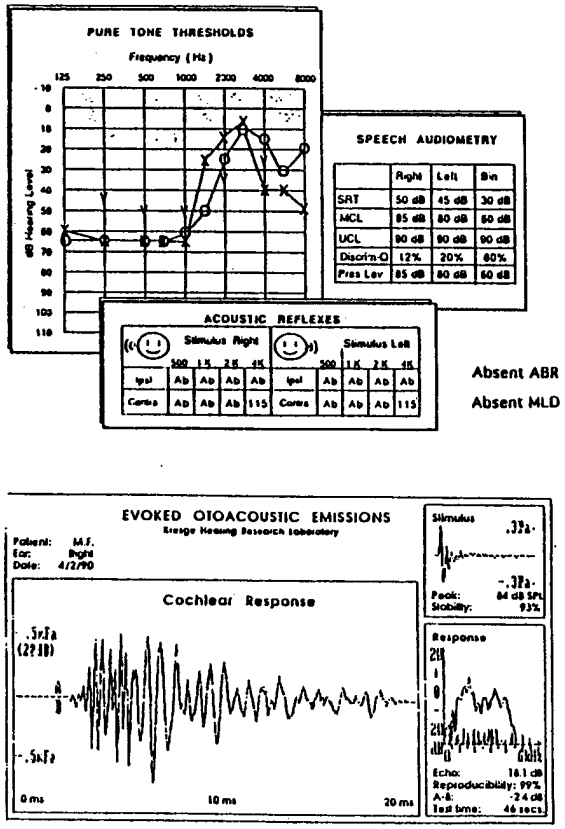


Figure 6–6. Patient with auditory neuropathy and low frequency loss who also has no ABR, no MLD, but normal otoacoustic emissions.

she would be an excellent candidate for hearing aids of one of two types—either a low-frequency emphasis aid with no insertion loss (Killion, Berlin, & Hood, 1984) or one of our body borne translators (Berlin et al., 1978). Her clinical complaint was that she simply could not understand speech in quiet or in noise, and also suffered from mild unsteadiness and poor coordination. She manages quite well in everyday life with the help of her husband, and runs a successful service business. We were not sophisticated enough at the time to recognize the signs that she was not a candidate for ordinary amplification. She tried the K-Bass (Killion et al., 1984) aid with the report that it helped heighten her awareness that someone was talking to her but it did not help her understand speech. We were puzzled at the time over the absence of an ABR to clicks or tone bursts around 2 kHz and sent her for a neurological workup. She was diagnosed as having an unspecified leukodystrophy, not MS, and has had no further clarification of her status since then, other than her disease resembles Charcot-Marie-Tooth syndrome but is slightly different (Starr & Picton, personal communication).

Audiological Findings

Her middle ear muscle reflexes were absent despite normal tympanometry. Also absent were any release from masking during MLD testing (Hirsh, 1948) and any synchronous ABR discharge, either to clicks or tone bursts around the zone of her normal hearing. Most surprising, however, were her unusually large and spectrally complex otoacoustic emissions which conventional wisdom predicted would be absent except in a narrow zone between 2 and 3 kHz. (Figure 6–6).

She showed complete absence of contralateral suppression of her otoacoustic emissions at any combination of click intensities and noise levels we used (ranging from 60 to 80 dB peak SP). Because of the absence of both the first wave of the ABR and the total absence of efferent suppression, we conclude that the primary neurons are not synchronous enough to activate an efferent outflow from the brainstem (Berlin et al., 1994).

Patient 2

This 40-year-old woman came to us originally carrying a diagnosis of Charcot-Marie-Tooth syndrome passed down from her father. Her sister is similarly afflicted and, as a matter of fact, is the next patient in the chapter. Figure 6–3 shows her audiogram, and Figure 6–4 shows the somewhat less severe appearing pure tone loss of her sister. It is reasonable to anticipate that Patient 2 would not have much of an ABR, MLD, or middle ear muscle reflex. But what is surprising is that she has norm

and robust otoacoustic emissions and absolutely no contralateral suppression. The evidence for her systemic neuropathy comes from the absence of sensory nerve response in her right arm, and slowed transmission time and reduced motor response of the right median nerve, with normal response of the ulnar nerve. She had no success with the hearing aids we had prescribed (before we observed her normal otoacoustic emissions data).

Patient 3

This 36-year-old woman, who is the sister of the previous patient, also shows no middle ear muscle reflexes, no ABR, no MLD, and large and robust otoacoustic emissions, a paradoxical finding again in view of her pure tone hearing loss (Figure 6–4). She too showed no contralateral suppression and had no success with any hearing aids we tried.

Patient 4

The first three patients all had abnormal audiograms for which one might prescribe hearing aids if one had no information about otoacoustic emissions. In stark contrast, Patient 4 has a nearly normal audiogram but no ABR, no middle ear muscle reflex response, and would have appeared to be deaf, if we had tested him only with ABRs (Figure 6–2). Since he was brought to us at age 12 with a complaint that he simply couldn't understand other people's speech, he was easy to test behaviorally. That's how we found that, despite his absent ABR, he had normal otoacoustic emissions, no efferent suppression, and no MLDs.

What Do the Outer Hair Cells Do?

Outer hair cells probably contribute to some form of mechanical or electrical amplification of low amplitude acoustic inputs or compression of high amplitude inputs. Among the most illuminating observations in this area are the Mossbauer studies (Ruggero, 1992) summarized in Figure 6–7. Here we see that for a 3 dB input signal at 9 kHz the hair cells reflect a 10,000× amplification. In contrast, when the input signal reaches 80 dB, the hair cells impart a little more than 10× amplification. It is clear that outer hair cells play an important part in whatever compression mechanisms operate in the normal ear. The gain functions of the K-Amp and ReSound hearing aids (Figure 6–8) are qualitatively similar to the gain functions Ruggero observed.

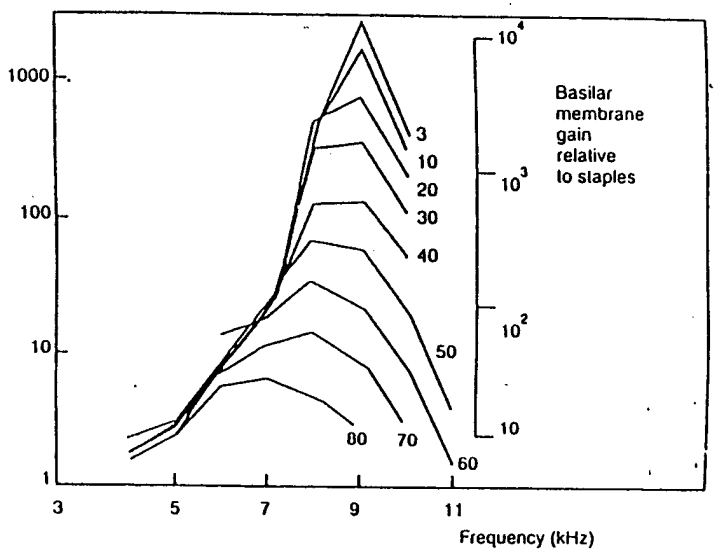


Figure 6–7. Mechanical responses of a chinchilla cochlea to tones. Note that at peak, this gain is more than 10,000 for the lowest stimulus level (3 dB SPL) but drops to values between 10 and 100 for sound levels typical of speech (60 dB SPL to 80 dB SPL). Taken with permission from Ruggero (1992).

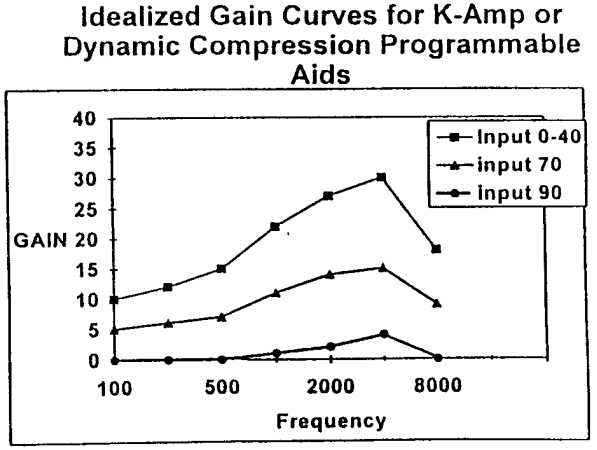


Figure 6–8. An idealized gain curve for K-Amp or dynamic compression programmable aids. Notice how

decreasing gain system with increasing input which compensates for the disruptive effects of recruitment on the complex speech signal. It is this expansion of the loudness of vowel peaks and loss of perceived consonant-vowel relationships in speech that will be the topic of the next presentation (Villchur, 1973, 1974).

SUMMARY AND CONCLUSIONS

Otoacoustic emissions and ABR, when used together, form a powerful combination, offering insight into preneural as well as neural function in the cochlea. If emissions are present, patients are *not* hearing aid candidates. Conversely, it is the *absence* of outer hair cell echoes, in the presence of robust and synchronous ABRs at high intensities, that suggests that patients with hearing losses from 20 to 70 dB HL are *good* candidates for dynamic compression aids. These aids compensate for the presumed loss of the low-level amplification ascribed to the outer hair cells, which bring faint sounds into smooth, broad-band, audibility.

Unfortunately, many people are living today with "yesterday's diagnosis": *You have "nerve deafness" and hearing aids will be of limited value.* The fact is that linear aids with restricted frequency responses and ordinary peak clipping simply drove many patients to take the aids off. The aids made vocalic segments of speech disproportionately loud and hence distorted to the listener who was hearing impaired. Because of their restricted frequency response, these aids also limited the quality and number of auditory cues patients could use to hear both in noise and in crowds the way people with normal hearing do.

The kernel message: it is no longer appropriate to fit a linear hearing aid to patients with hair-cell-based losses from 20 to 75dB HL. High fidelity dynamic compression aids that make low-level signals uniformly and smoothly audible are useful answers to the sharp and uneven loudness growth of segments of the speech code for people with recruitment.

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APPENDIX # 11
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Bobbin and Berlin

3

Suppression of Otoacoustic Emissions in Normal Hearing Individuals

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INTRODUCTION

The recent discoveries of evoked otoacoustic emissions (Kemp, 1978) and of outer hair cell motility (Brownell, Bader, Bertrand, & deRibaupierre, 1985) have contributed to a significant increase in the understanding of the role of cochlear micromechanics in the processing of auditory stimuli. Otoacoustic emissions are low-level sounds that emanate from the cochlea and can be recorded from the external ear canal using sensitive low-noise microphones. They are associated with nonlinear processes present in the normal cochlea and enhanced sensitivity and tuning of the auditory system. The origin of otoacoustic emissions is ascribed to processes associated with the mechanical motion of the outer hair cells, thought to be controlled through the efferent auditory pathways via the olivocochlear system (Kemp, 1978; Kemp & Chum, 1980; Probst, Lonsbury-Martin, 1991; Norton & Widin, 1990).

There are two broad categories of otoacoustic emissions (OAEs): spontaneous and evoked (Table 3–1). Spontaneous otoacoustic emissions, or SOAEs, by definition occur spontaneously, requiring no external stim-

TABLE 3–1. Classification of otoacoustic emissions.

Category	Type	Stimulus
Spontaneous		None
Evoked	Transient	Clicks or tonebursts
	Distortion Product	Pairs of pure tones
	Stimulus Frequency	Swept pure tones

ulus to elicit a response. In contrast, evoked emissions fall into three classes, each requiring a different type of external stimulus. Transient evoked otoacoustic emissions (TEOAEs) are obtained in response to brief stimuli such as clicks or tonebursts, distortion product otoacoustic emissions (DPOAEs or DPEs) are produced by pairs of pure tones, and stimulus frequency emissions (SFOAEs) are generated by presentation of continuous tonal stimuli. Otoacoustic emissions have received extensive attention in human and animal research and several reviews are available (e.g., Glattke & Kujawa, 1991; Martin, Probst, & Lonsbury-Martin, 1990; Probst et al., 1991). Clinical applications have focused on TEOAEs and DPOAEs in the evaluation of cochlear function, screening for hearing loss in newborns, and monitoring changes in auditory function.

Emissions are not only valuable in analyzing the integrity of individual ears; they can also be used to evaluate interactions between the two ears by studying emission suppression following presentation of additional stimuli to the same, opposite, or both ears. A number of studies have described suppression of spontaneous and transient emissions in humans by contralateral acoustic stimuli (Berlin, Hood, Cecola, Jackson, & Szabo, 1993a; Collet et al., 1990; Grose, 1983; Schloth & Zwicker, 1983; Mott, Norton, Neely, & Warr, 1989; Rabinowitz & Widen, 1984; Ryan, Kemp, & Hinchcliffe, 1991; Veuillet, Collet, & Duclaux, 1991). These suppression effects observed in humans are consistent with suppression of both cochlear emissions and auditory nerve activity observed in animals (e.g. Buno, 1978; Liberman, 1989; Mountain, 1980; Puel & Rebillard, 1990).

Anatomical and physiological evidence supports the interdependent function of the two ears mediated through the efferent neural pathways which link one side of the auditory system to the other side via the medial and lateral components of the olivocochlear system (Warr & Guinan, 1978; Warr, Guinan, & White, 1986). The medial olivocochlear components terminate primarily on the outer hair cells, whereas the lateral olivocochlear components terminate mainly on primary auditory neurons at the base of the inner hair cells. Outer hair cell activity is believed to be modified through the medial efferent connections. Function of these pathways can be studied objectively and noninvasively in animals and in humans using suppression of otoacoustic emissions.

Suppression of transient evoked otoacoustic emissions has been a subject of study at Kresge Hearing Research Laboratory for several years. Research has focused on understanding the physiology of suppression of emissions, definition of the normal characteristics of suppression, parametric study of stimulus and external factors that affect suppression, the development of methodology that leads to efficient and reliable recording and analysis of suppression, and delineation of clinical applications. In this chapter, we focus on the characteristics of suppression in individuals with normal auditory function and describe the effects of stimulus and noise characteristics on suppression and the definition of suppression effects through specialized analysis techniques. This chapter is followed by a companion chapter which describes clinical patients who show no suppression of emissions and discusses ways in which suppression information can be used in the management of hearing loss.

Subjects

Subjects participating in the following studies all had normal hearing in both ears, defined as thresholds of 15 dB HL or better for the frequency range of .25 through 8 kHz. Middle ear measures (tympanograms and ipsilateral and contralateral acoustic reflex thresholds) were within normal limits and subjects had no history of neurological abnormality.

General TEOAE Recording Methods

For the studies described here, transient-evoked otoacoustic emissions were obtained using an Otodynamics ILO88 otoacoustic emissions system. Stimuli were 80-microsecond clicks which were nonlinear in our early studies, but linear in more recent studies. Nonlinear click trains present three stimuli of like phase followed by a fourth stimulus both opposite in phase and 10 dB higher in intensity. This type of stimulus paradigm is desirable in general emissions test situations to reduce stimulus artifact while maintaining a quantifiable otoacoustic emission. However, use of nonlinear click trains affects the true amplitude of the emission and thus makes absolute quantification of suppression effects difficult. Thus, the use of linear clicks (all clicks of like phase) is both desirable and possible since lower intensity stimuli are generally used in suppression studies. Suppressor (masker) stimuli are generated either externally (when contralateral noise is presented simultaneously) or internally by the ILO88

system (in a forward masking paradigm). When externally generated, noise levels are continually monitored with a probe microphone.

Three control (without contralateral noise) and three experimental (with contralateral noise) test conditions are alternated and averaged separately for each subject prior to analysis in accordance with the method suggested by Collet et al. (1990). Averages of 260 click trains of 4 clicks each are obtained for a total of 1,040 stimuli per condition, unless otherwise noted. Responses are accepted when the stimulus stability exceeds 80% and the response reproducibility exceeds 70%. Stimulus stability represents a comparison of stimulus level recorded in the ear canal at the beginning of the test to the level of the stimulus monitored throughout the test acquisition period. Response reproducibility represents the correlation of averages of half of the sweeps which are stored in one computer buffer and the other half of the sweeps stored in another memory buffer. These two averages are acquired by interleaving sweeps between two computer memories during the acquisition of an emission. A typical evoked otoacoustic emission obtained from a female subject with normal hearing using the ILO88 System is shown in Figure 3–1. In this case, the overall amplitude of the emission is 12.9 dB and the response reproducibility is 98%. The stimuli were linear clicks which were monitored at

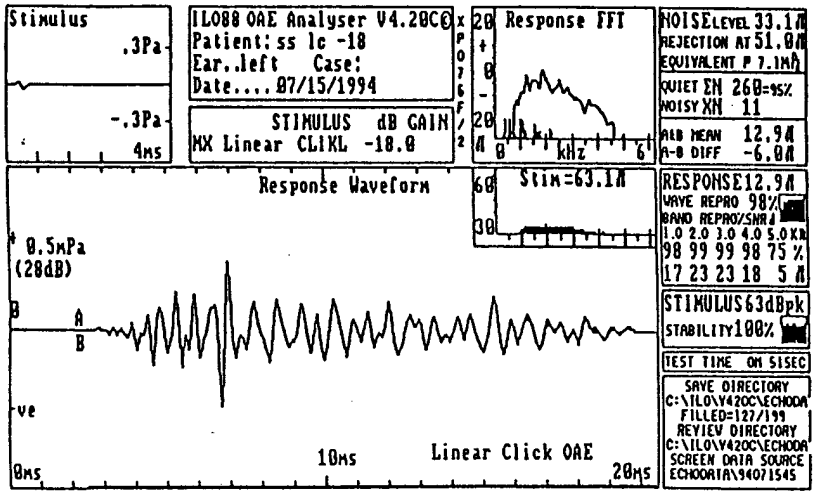

Figure 3–1. Transient Evoked Otoacoustic Emissions (TEOAEs) obtained from a normal subject using linear clicks.

an intensity of 63 dB peak sound pressure in the ear canal, and the stimulus stability during the test was 100%.

Early Studies

In our early studies of suppression, transient otoacoustic emissions were obtained using nonlinear clicks at 80 dB peak SP (sound pressure). The emissions were suppressed by either pure tones (at octave intervals from 250 to 4kHz), narrow band noises (centered at octave intervals from 250 to 4kHz), or broad band noise presented simultaneously to the contralateral ear. Results indicated greater suppression with increasing masker levels, greater effects with narrow-band noises than with pure tones, and greater suppression effects for lower frequency than higher frequency maskers. The details of these studies were reported in Berlin et al. (1993a).

In all of our studies, we reported suppression effects as dB differences in emission amplitude between conditions with and without noise rather than as equivalent dB, as Collet and colleagues have used (Collet et al., 1990; Veuillet et al., 1991). Although the differences observed in overall emission amplitude between conditions without and with noise appear small (on the order of 1 or 2 dB), they are quite consistent. We also observed that suppression effects are greater in certain time periods, which led us to the development of a method to analyze suppression of emissions in greater detail.

Development of Data Analysis Techniques

As we sought to refine our understanding of suppression, one of the authors (HW) developed an analysis program to quantify changes in transient evoked otoacoustic emissions obtained under varying test conditions (Wen, Berlin, Hood, Jackson, Hurley, 1993). The Kresge EchoMaster Program (current version 3.1) is compatible with the Otodynamics ILO88 file structure and allows detailed comparisons of root-mean-square (RMS) amplitude, cross correlations, and time delays across the entire time window or in selectable time periods. Frequency domain data can also be obtained using fast Fourier transforms (FFT) and selectable windowing functions to quantify emission spectra. The EchoMaster program provides comparisons of (1) two individual emissions, (2) the means of two groups of emissions with up to 60 emissions in each group, and (3) an individual emission with its estimated background noise. An example of the data obtained from the EchoMaster analysis is shown in Figure 3–2.

Prior to addition of data, like conditions (i.e., the three conditions without noise and the three conditions with noise) are reviewed for data

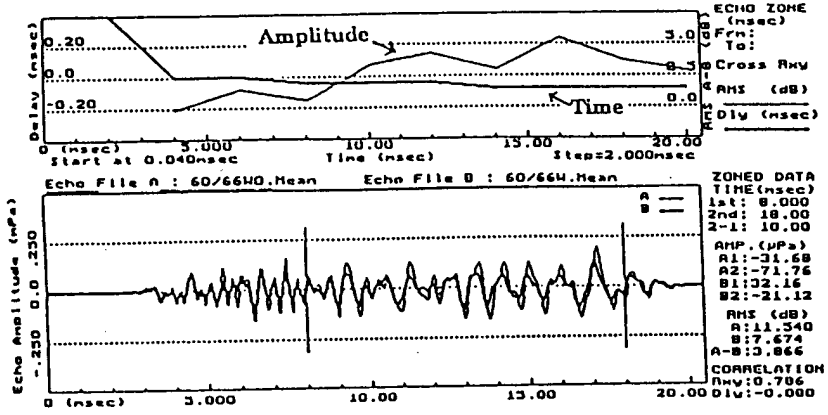

Figure 3–2. Analysis method for quantification of suppression using the Kresge EchoMaster program. Emissions obtained without (thin line) and with (thick line) are shown in the bottom portion. The top portion shows amplitude and time differences between the without and with conditions in 2-ms intervals.

consistency. Mean data are obtained for emissions from like conditions (i.e., without or with noise) only within subjects, and each subject's emissions are compared only with his or her own emissions. Once emission suppression is quantified for individual subjects through the EchoMaster analysis, we then can compare data across subjects.

CHARACTERISTICS OF TEOAE SUPPRESSION

What Are the Characteristics of Suppression in Normal Individuals?

Suppression of TEOAEs is characterized as a reduction in amplitude and/or a time change or phase shift (e.g., Berlin et al., 1993a; Collet et al., 1990; Ryan et al., 1991; Veuillet et al., 1991). Subjects vary in the amount of suppression, although in our experience all normal subjects show amplitude decreases with noise in some time periods. Some subjects demonstrate suppression of as much as 5 to 7 dB. As the intensity of the contralateral noise increases, the amount of suppression increases and time periods involved tend to broaden.

Suppression also is demonstrated as a change in the time of occurrence of peaks or zero crossings in the emissions, particularly at later time periods. Introduction of contralateral noise causes a decrease in the latency of peaks relative to the stimulus, or a "negative" time delay. This can be seen in the lower tracing of Figure 3–2 where the peaks in the suppressed condition are slightly earlier, particularly in later time periods. As the intensity of the contralateral noise increases, earlier time periods tend to be involved as well for both amplitude and time differences (Hood, Hurley, Wen, Berlin, & Jackson, 1993).

Test-retest reliability analyses have been incorporated into many of our studies of suppression. Results indicate good repeatability of suppression effects for both amplitude and time within subjects as well as consistent group effects (e.g., Hood et al., 1993).

Is Suppression Greater in Certain Time Periods?

A consistent observation throughout our studies is the occurrence of maximal suppression in the 8- to 18-ms time period which is consistent with other reports (e.g., Collet et al., 1990). Analysis by 2 ms intervals, as shown in Figure 3–3, indicates that suppression varies across time and that the greatest amplitude suppression and time delay changes occur in the time period between 8 and 18 ms. In this figure, data obtained from the EchoMaster analysis of a series of normal subjects have been combined to examine mean suppression effects on amplitude (left panel) and time (right panel). Very little suppression is observed from 2 to 8 ms in either the amplitude or time graphs. Effects across this group of subjects increase with time and are greatest in the 8- to 18-ms regions. Boxes outline the time window that is used to quantify suppression effects in our subsequent studies.

The "Echo in dB" value from the ILO88 system describes the characteristics of an entire emission as a single value. Because suppression effects are greater in some regions than in others, a single value cannot adequately represent the maximal effects or define time regions containing the greatest amount of suppression. In fact, an overall aggregate number will underestimate the suppression effect in most subjects. Thus, we recommend point-by-point analysis of suppression at 1-, 2-, or 3-ms time intervals to identify time regions of maximum effect and more precise quantification of suppression.

An example of a subject with minimal suppression when analyzed with the overall ILO88 number is shown in Figure 3–4. Although this single number might suggest the lack of a suppression effect, this subject shows substantial suppression between 10 and 12 ms. In addition, some subjects show greater amplitude changes while other subjects may show greater temporal changes.

Do Intensity Levels of the Click and Noise Affect Suppression?

We reasoned that because otoacoustic emissions are associated with cochlear processes occurring in response to low-intensity stimuli, it is

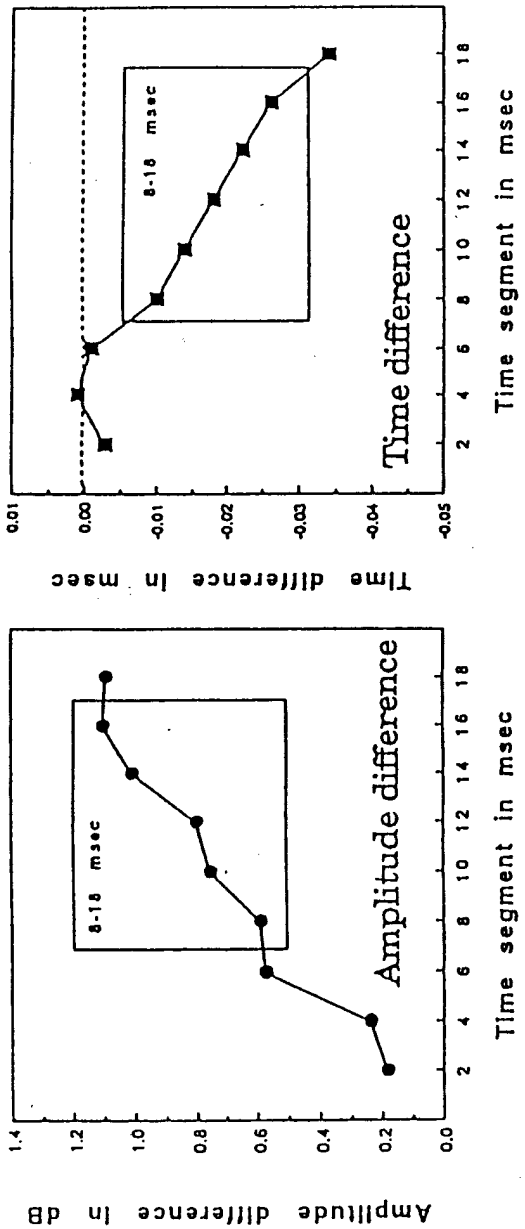

Figure 3–3. Mean data from a group of normal subjects show maximal amplitude (left) and time (right) differences in the 8- to 18-ms time window.

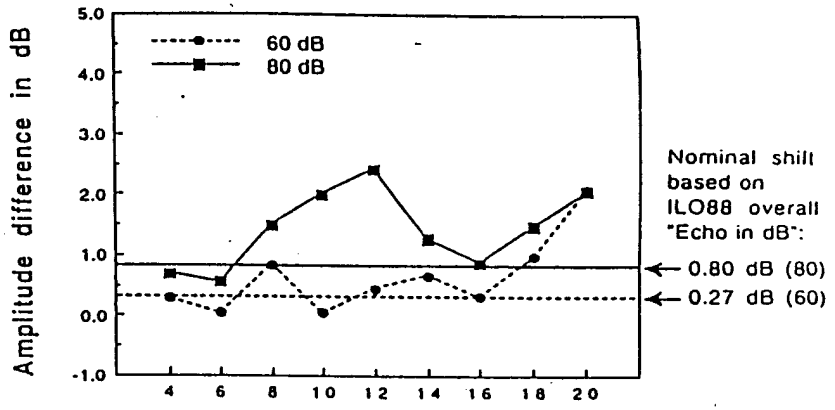

Figure 3–4. Some normal subjects who appear to have little contralateral suppression calculated in overall dB by the ILO88 system show clear amplitude changes in isolated time periods. Shown are data for one subject who shows less than 1 dB of suppression in the aggregate echo but more than 2.4 dB suppression in the 10–12 ms time period.

possible that fainter click and noise levels may be optimal for observing suppression effects. In one study, we varied the intensity of both linear clicks and contralaterally presented continuous white noise to determine the optimal click and noise levels that would yield the greatest contralateral suppression effect (Hood, Berlin, Hurley, Cecola, & Bell, 1994).

Comparison of suppression for linear clicks of peak sound pressures from 50 to 70 dB and contralateral white noise from 10 dB below to 10 dB above the click peak SP showed that the amount of suppression is dependent on both the level of the suppressor noise and the level of the stimulus eliciting the emission. Suppression increased systematically as the level of the noise was increased. Maximum suppression was reached with clicks of 55 dB peak SP and above (Figure 3–5). The observation of a decrease in suppression for 65 dB peak SP clicks suggests the possibility of different "low" and "high" level processes affecting suppression, depending on the stimulus and noise intensities.

The occurrence of greater suppression for some lower rather than higher intensity stimuli described here also reduces concern about contamination of suppression by either acoustic crosstalk or acoustic reflexes, both of which may contribute at high intensities but could not have a greater effect at lower than higher intensities.

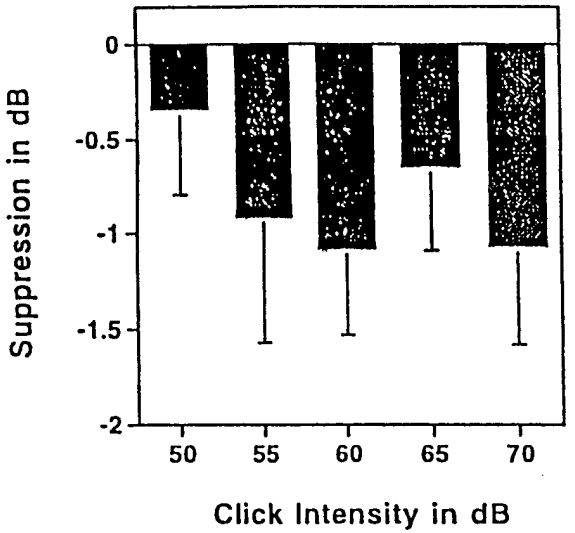

Figure 3–5. Effects of stimulus and suppressor intensity on suppression. The amount of suppression increases with increasing suppressor intensity, but is greater for some lower intensity stimuli.

BINAURAL, IPSILATERAL, AND CONTRALATERAL SUPPRESSION

The olivocochlear system can alter outer hair cell activity when activated by contralateral, ipsilateral, or bilateral stimuli. Although most experiments on suppression of otoacoustic emissions have involved contralateral simultaneous stimuli, Berlin, Hood, Hurley, Wen, and Kemp (1995) extended TEOAE suppression studies to the presentation of ipsilateral and bilateral suppressors as well as contralateral suppressors. In order to accomplish this, it was necessary to develop a forward masking paradigm which allowed temporal separation of the suppressor and the emission evoking click. This was accomplished through the assistance of Dr. David Kemp, who provided the software necessary to complete a forward masking paradigm using the Otodynamics ILO88 system.

Forward Masking Paradigm

In a forward masking paradigm, the masker precedes the test stimulus in time. By separating the masker and stimulus in time, acoustic interaction

of the two signals is minimized. Even though a masker precedes the test stimulus, psychophysical studies have shown that a masking effect persists for a short time after the cessation of the masker.

To study the effects of binaural, ipsilateral, and contralateral noise on suppression we collected TEOAEs in a forward masking paradigm using linear clicks at 65 dB peak SP and a 65 dB SPL white noise masker that was 400 ms in duration (Figure 3–6). Time separations between the offset of the noise and the onset of the first click of a four-click series were varied from 1 to 200 ms. Because the ILO88 presents stimuli in groups of four, the three clicks following the initial stimulus were each separated by an additional 20 ms. Thus, only the first click in the series was masked according to these delay times, a limitation that we are currently correcting with another paradigm which allows presentation of a single stimulus following the masker.

Do Ipsilateral and Bilateral Noise Stimuli Yield Results Similar to Contralateral Noise?

Binaurally presented noise resulted in significantly greater suppression than ipsilateral or contralateral noise (Figure 3–7) and contralateral noise was the least effective suppressor (Berlin et al., 1995). Consistent with previous studies, the greatest suppression occurred between 8 and 18 ms following stimulus onset. Mean maximal suppression effects were on the order of 3.0 to 3.5 dB for binaural noise, 1.5 to 2.0 dB for ipsilateral noise, and 1.0 to 1.5 dB for contralateral noise.

Does the Time Separation Between the Noise Offset and the Click Affect Suppression?

The literature from behavioral studies of forward masking indicates that the effectiveness of a masker decreases as the time separation between

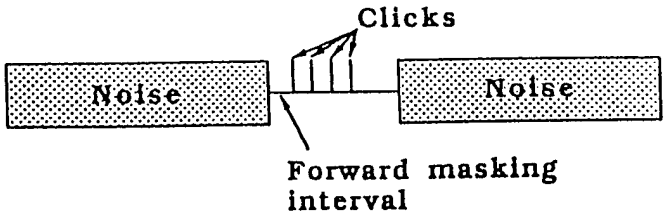

Figure 3–6. Diagram of a forward masking paradigm used to evaluate suppression of emissions.

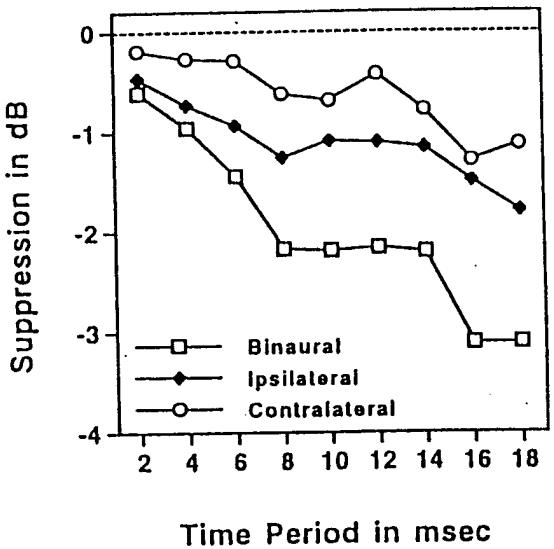

Figure 3–7. Comparison of suppression with ipsilateral, contralateral, and binaural noise. Binaural stimulation is more effective than either ipsilateral or contralateral noise.

the offset of the masker noise and the onset of the stimulus increases. Consistent with behavioral studies, the amount of emission suppression decreased systematically with increasing time separations between the masker and the click train (Berlin et al., 1995). One ms separation yielded the most suppression, and little or no suppression was observed when the noise and clicks were separated by 100 to 200 ms. It should be noted that these suppression effects may underestimate the actual suppression due to the additional time delay between the noise and the second, third, and fourth clicks in the train. When the time separation between a masker noise and a click is less than 10 ms, it is also possible that the noise itself will produce an emission that will contaminate the target response (Tavartkiladze et al., 1995). Thus, a time separation of 10 ms between the noise offset and stimulus onset appears to yield a suppression effect with minimized contamination.

Does Noise Duration Affect Suppression?

Liberman and Brown (1986) first showed that stimulation of 50 to 500 ms is required to obtain responses from olivocochlear neurons. For this reason, we investigated the effects of noise duration in a forward-masking paradigm on TEOAE suppression (Hood, Berlin, Wakefield, & Hurley, 1995). We compared broad-band noise durations from 80 to 640 ms (at 65 dB SPL) presented prior to the onset of 65 dB peak SP linear clicks. Increases in noise duration yielded progressively greater suppression through 400 ms with no significant increase in suppression for noise durations from 400 to 640 ms. These data extend to humans the observations made by Liberman and Brown (1986) that a minimum stimulus duration is necessary for activation of the efferent system.

Potential Problems and Other Considerations

Several factors have the potential to contribute to a reduction in emissions when noise is introduced. These include activation of the middle ear muscle reflexes and acoustic crosstalk or crossover of sound through the head. However, several factors reduce the possibility that either of these factors plays a major role in the observed suppression effects. Our data showing greater suppression effects for lower intensity stimuli minimize the potential role of the middle ear muscle reflex and crossover, as effects would be expected to increase with intensity if they represented either of these phenomena. Acoustic crossover is also unlikely because we used insert earphones with 60–90 dB interaural attenuation (Killion, Wilbur, & Gudmundsen, 1985). In addition, both our group and Collet and his colleagues have observed suppression in patients who lack stapedial muscle function due either to Bell's Palsy or stapedial tendon section during stapedectomy.

It is important to monitor click and noise levels during evaluation because these levels vary greatly across ears. Consistent with Collet and his colleagues, we also recommend use of linear rather than nonlinear clicks and presentation levels in the range of 55 to 60 dB peak SP in order to avoid the potential contaminants discussed.

Clinical Application

Based on our studies, we measure suppression of transient evoked otoacoustic emissions clinically using 55 or 60 dB peak SP linear clicks and noise levels of 55 to 65 dB SPL. We interleave three "without noise" and three "with noise" conditions, analyze results using the Kresge EchoMaster software, and focus on the 8- to 18-ms time period. In patients of interest, we present bilateral, ipsilateral, and contralateral noise in a forward masking paradigm. We believe that suppression of otoacoustic emissions provides insight into function of the efferent system and the interactions between the afferent and efferent pathways which allow us to distinguish

central from peripheral hearing losses and manage both types of hearing disorders more accurately (e.g., Berlin et al., 1993b; Williams, Brooke, & Prasher, 1994).

SUMMARY

The series of studies summarized in this chapter provide information about some of the characteristics of suppression of transient evoked otoacoustic emissions in adults with normal hearing. These characteristics of suppression can be summarized as follows:

1. Suppression is characterized by amplitude decreases as well as time shifts of emission peaks.
2. Suppression is greatest in the 8- to 18-ms time period.
3. Some normal subjects who appear to have little or no suppression when a single value is calculated to represent the entire 20.48 ms period show clear suppression in the 8- to 18-ms range.
4. As the intensity of the suppressor noise increases, suppression amplitude and time differences increase.
5. Suppression is greater for lower intensity stimuli than for higher intensity stimuli.
6. Suppression is greater for binaural noise than for ipsilateral or contralateral noise.
7. Suppression is greatest for time separations of less than 10 ms and for noise durations greater than 400 ms.
8. Test and retest comparisons show that suppression effects are repeatable.
9. Suppression of otoacoustic emissions is useful clinically in evaluating and managing patients with central and peripheral hearing losses.

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APPENDIX # 12
DAMD17-93-V-3013
Bobbin and Berlin

126 Chronic low-level noise exposure alters distortion product otoacoustic emissions

R.A. Skellett, J.R. Crist, M. Fallon, R.P. Bobbin (Kresge Hearing Research Lab., LSUMC, New Orleans)

Chen *et al.* (*Hear. Res.* in press, 1995) recently reported an altered response to the application of ATP in outer hair cells (OHCs) isolated from guinea pigs continuously exposed for 10 or 11 days to a 65 dB SPL (A-scale) narrowband noise (1.1 - 2 kHz). The primary goal of the present study was to test the hypothesis that the continuous low-level noise used by Chen *et al.* alters cochlear function. Cubic ($2f_1$-f_2) and quadratic (f_2-f_1) DPOAEs, as well as, the amount of contralateral suppression of DPOAE amplitudes were chosen for study. Responses were recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles. The animals had either been exposed to the low-level noise for 3 or 11 days or not exposed at all (n = 13 animals per group). Results demonstrate that this noise induces frequency-dependent and very localized reductions in $2f_1$-f_2 DPOAE input-output functions. However, the f_2-f_1 DPOAE input-output functions and the time-varying amplitude characteristics of f_2-f_1 DPOAE appear to be insensitive to the noise exposure. No noise-related changes were found in the amount of contralateral suppression between the different exposure groups, with the exception of one unexplainable data point (f_2-f_1 DPOAE = 0.5 kHz; day 3) where it was reduced. The $2f_1$-f_2 DPOAE amplitude alterations lend support to the conclusions of Chen *et al.* that chronic low-level noise exposure induces molecular changes in the OHCs which may, in turn, alter cochlear function.

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Abstracts of the 19th Midwinter Research Meeting, Association for Research in Otolaryngology, St. Petersburg Beach, Florida, Abstract 126, Feb 4-8, 1996

APPENDIX # 13
DAMD17-93-V-3013
Bobbin and Berlin

136 "Conditioning" the auditory system with continuous *vs.* interrupted noise of equal acoustic energy: Is either exposure more protective?

R.A. Skellett, M. Fallon, R.P. Bobbin (Kresge Hearing Research Lab., LSUMC, New Orleans)

The temporal pattern of noise exposure is an important factor which affects both the magnitude of cochlear damage and the pattern of auditory sensitivity change over time. Continuous noise exposure is more damaging to the cochlea than an interrupted exposure of equal energy (Bohne *et al., Ann Otol. Rhinol. Laryngol.* 94: 122, 1985; Fredelius and Wersäll, *Hear. Res.* 62: 194, 1992). Continuous noise exposure results in a pattern of sensitivity change referred to as an asymptotic threshold shift (ATS; Carder and Miller, J. *Speech Hear. Res.* 15: 603, 1972). Interrupted noise exposure results in a progressive resistance to threshold shift known as "toughening" (Clark *et al., J. Acoust. Soc. Am.* 82: 1253, 1987; Subramaniam *et al., Hear. Res.* 52: 181, 1991). Both exposure schedules have been used in "conditioning" experiments and both have been effective in providing protection against subsequent traumatizing noise (Canlon *et al., Hear. Res.* 34: 197, 1988; Campo *et al., Hear Res.* 55: 195, 1991). The purpose of this investigation is to determine if differences exist in the amount of protection afforded by prior "conditioning" of the auditory system with continuous or interrupted noise of equal acoustic energy.

Guinea pigs were "conditioned" with an octave band of noise (1-2 kHz) for 24 hours per day for 11 days at 89 dB SPL (n=14) or for 6 hours per day for 11 days at 95 dB SPL (n=14). After a one week rest period, both groups were continuously exposed for 3 days to the same noise spectrum at 105 dB SPL. A third group of animals (n=14) was only exposed to the high-level exposure. All animals were given 4 weeks to recover from the high-level exposure. Cubic ($2f_1$-f_2) DPOAEs were then recorded in urethane-anesthetized guinea pigs with sectioned middle ear muscles.

Preliminary results suggest that an interrupted "conditioning" noise may provide more protection against subsequent damaging exposures than a continuous exposure of equal acoustic energy. However, the results obtained thus far are not conclusive and require further experimentation to determine if statistically significant differences exist between the two exposure groups.

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APPENDIX # 14
DAMD17-93-V-3013
Bobbin and Berlin

526 ATP does not generate a current response in outer hair cells of rat cochlea

C. Chen, C.S. LeBlanc, R.P. Bobbin (Kresge Hearing Research Lab., LSUMC, New Orleans)

Increasing evidence suggests that ATP may act as a neurotransmitter and/or modulator in the mammalian cochlea (Kujawa *et al. Hear. Res.* 78:181, 1994). Extracellular application of ATP, related agonists, and ATP antagonists significantly affect cochlear potentials in guinea pig. In isolated outer hair cells (OHCs) of guinea pig, ATP depolarizes the cell membrane by inducing non-selective cation currents. However, nothing is known about the effect of ATP on rat OHCs. The purpose of the present study was to compare the ATP response in OHCs from guinea pig to the ATP response in OHCs from rat. The whole-cell configuration of the patch-clamp technique was used. OHCs were isolated from pigmented guinea pigs and Sprague Dawley rats. Extracellular application of 100 μM ATP or ATP-γ-S induced a large inward current in OHCs of guinea pig recorded at -60 and -100 mV. The same concentration of ATP or ATP-γ-S did not induce any detectable response in rat OHCs at -60 mV or at -100 mV. However in these same rat OHCs, 100 μM ACh induced an inward current at -100 mV and an outward current at 0 mV. When K^+ was substituted by N-methyl-glucamine (NMG^+) in the pipette solution, no current was induced in rat OHCs by ATP. Adenosine, AMP and ADP did not produce a response in rat or guinea pig OHCs. In both rat and guinea pig, ATP produced a very large inward current in supporting cells closely associated with the OHCs. Our results suggest that there may be no or very few ATP receptors in OHCs of rat cochlea. This is in contrast to guinea pig cochlea where ATP receptors on OHCs appear to be large in number. On the other hand, supporting cells in both rat and guinea pig seem to have a similar number and type of ATP receptors.

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