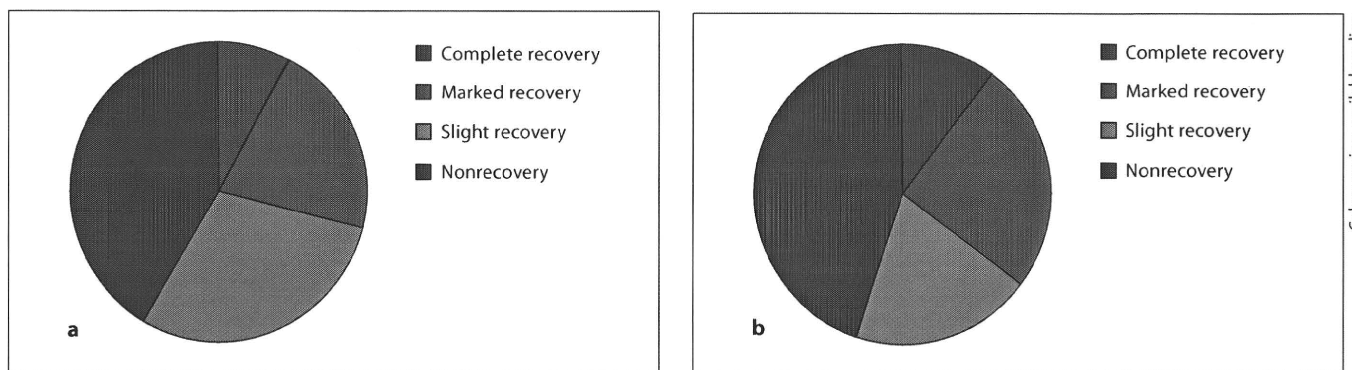


Fig. 2. a Hearing recovery results in group S. **b** Hearing improvement in patients with an initial mean hearing level <90 dB.

Side Effects

There was transient vertigo of about a minute associated with the injections. Some patients had tolerable pain after the injection for a minute. No patients had otitis media. The perforations were closed spontaneously or with a chitin sheet patch in most patients. For 12 patients, autologous serum ear drops and/or basic fibroblast growth factor with a chitin sheet patch therapy in the outpatient clinic [Kakehata et al., 2008] were performed and the perforations were closed successfully except in 1 patient. Small perforations remained in 2 patients. One patient underwent myringoplasty.

Discussion

Recently, there has been increased interest in IT administration of steroids for the treatment of ISSNHL, whether as an initial or salvage treatment [Parnes et al., 1999; Gianoli and Li, 2001; Guan-Min et al., 2004; Battista, 2005; Lautermann et al., 2005; Dallan et al., 2006; Kakehata et al., 2006; Haynes et al., 2007; Kilic et al., 2007; Plaza and Herraiz, 2007; Van Wijck et al., 2007; Ahn et al., 2008; Battaglia et al., 2008; Han et al., 2009; Hong et al., 2009; Plontke et al., 2009; Dallan et al., 2010; Kara et al., 2010], since ITS seems to be a potent alternative treatment option to systemic steroid therapy. As a first-line treatment, several clinical studies reported efficacy of combination therapies of ITS with systemic steroid therapy [Battista, 2005; Lautermann et al., 2005; Ahn et al., 2008; Battaglia et al., 2008]. However, there has been no ITS treatment protocol that seems to be superior [Hu and Parnes, 2009]. Thus, before discussing the efficacy of combination therapy, it seems necessary to elucidate a preferable ITS treatment protocol without concurrent or previous treatments. Here, we performed daily short-term IT DEX treatment on fresh ISSNHL patients without concurrent treatments in order to investigate the efficacy of ITS alone.

An ideal treatment of ISSNHL should have a high cure rate as well as a high rate of response. To improve the cure rate is especially important because patients are not satisfied, even if their hearing level is improved by 30 dB, if it does not improve to near the hearing level of the unaffected ear. In this study, the rate of response and the cure rate of daily short-term IT DEX administration alone as an initial treatment reached 95 and 63%, respectively. This cure rate is higher than that of IT DEX treatment without concurrent systemic steroids in the recently published studies. Battaglia et al. [2008] reported a cure rate of 29% (5/17) by IT DEX therapy of 3 weekly injections. In their study, a high-dose oral prednisone treatment (tapering from 60 mg for 14 days; total 660 mg) and a combination therapy (IT DEX plus high-dose oral steroids) were also attempted. The cure rate of the oral steroid therapy and the combination therapy was 17% (3/18) and 63% (10/16), respectively. Battaglia et al. [2008] advocated the efficacy of the combination therapy over the systemic high-dose steroid therapy. Our cure rate was higher than that of the IT DEX therapy of 3 weekly injections and comparable to that of the combination therapy. This suggests that daily administration through LAM for 8 days is more effective than 3 weekly injections. In addition, in patients who did not have a profound hearing loss, our cure rate of 85% is higher than that of the combination therapy (63%). In the profound hearing loss group, the mean hearing improvement reached 37.8 dB, which was not statistically significantly different compared to the 40.5 dB in the other group. However, the cure rate was

low (17%) compared to that of 66% (deduced from figure 3 in Battaglia et al. [2008]) in the combination therapy, which might suggest a limitation of the IT DEX therapy in this form for patients with a profound hearing loss. This may also suggest that there are other pathological conditions in the case of profound hearing loss which cannot be reached by ITS treatment.

On the other hand, Ahn et al. [2008] reported an additional effect of IT DEX (3 injections on days 1, 3 and 5) through a 25-gauge spinal needle on oral methylprednisolone therapy (tapering from 48 mg for 14 days) in a study with a larger number of subjects. The cure rate by the combination therapy of IT DEX plus oral methylprednisolone was 25% (15/60), which did not show significant improvement compared to the oral methylprednisolone alone (27%, 16/60).

Battaglia et al. [2008] and Ahn et al. [2008] used a treatment protocol of 3 transtympanic injections of DEX on infrequent days. In most studies, steroids were administered by transtympanic injection through a fine needle under local anesthesia at 1–5 injections over 1–3 weeks [Hu and Parnes, 2009]. Recently, Kara et al. [2010] reported a high cure rate of 48% (14/29) by 5 transtympanic injections of IT DEX on 5 consecutive days as an initial treatment. Taking into account our high cure rate by 8 injections on 8 consecutive days, daily application of ITS may be more effective than infrequent application.

Recent studies on cochlear pharmacokinetics with local ear drug delivery revealed that after the end of the 30-min application, the concentration in the base of the scala tympani rapidly declines due to clearance from the cochlea and diffusion into other compartments and apical regions [Plontke et al., 2007]. To conquer this drawback, several delivery methods have been devised for the sustained application of the drug to the RW membrane, such as the Silverstein MicroWick [Silverstein et al., 1997] or the MicroCatheter [Kopke et al., 2001; Plontke et al., 2009]. However, the latter is no longer available because the FDA removed it from the market. With the Silverstein MicroWick, the patient can apply the steroid several times a day. In our previous study, we speculated that our high response rate and the degree of improvement might be due mainly to the delivery methods used and/or the frequency of the applications. The delivery method with a wide opening to the mesotympanum assures the certain filling of the mesotympanum with the treatment agent and allows the air to escape from the mesotympanum, permitting the treatment agent to contact the RW membrane. The high cure rate as an initial treatment in this study might support this speculation.

As a salvage treatment, improvement of more than 10 dB was achieved in 58% and the cure rate was 8% with successful treatment in 29%. The mean improvement was 16.8 dB. In the responder group, the mean improvement reached 30.1 dB. There are a number of studies of ITS as a salvage treatment. Reported response rates were between 27.5 and 73.6% [Parnes et al., 1999; Chandrasekhar et al., 2000; Gianoli and Li, 2001; Guan-Min et al., 2004; Dallan et al., 2006; Haynes et al., 2007; Kilic et al., 2007; Plaza and Herraiz, 2007; Van Wijck et al., 2007; Plontke et al., 2009; Dallan et al., 2010].

Initial severity of hearing loss is one of the known prognostic factors. Although there were no significant differences between the initial and salvage groups regarding the hearing level before the IT DEX treatment (77.7 ± 18.2 vs. 74.6 ± 15.3 dB) ($p < 0.01$), the hearing level after the treatment (38.8 ± 22.3 vs. 57.8 ± 21.8 dB) and gain of improvement (39.7 ± 18.4 vs. 16.8 ± 21.6 dB) were significantly higher in group I ($p < 0.01$). One of the reasons for these better results is the shorter duration between the onset and IT DEX treatment (4.8 ± 5.0 vs. 15.3 ± 6.4 days; $p < 0.01$). However, between the patients with successful treatment and those with no response in group S, the difference in the mean duration between onset of symptoms and IT DEX treatment (12.3 ± 3.5 vs. 16.5 ± 7.1 days) was not statistically significant, although the patients starting IT DEX treatment later than 19 days after onset did not have successful outcomes, suggesting the therapeutic window of this treatment. It is also likely that group S may include those with poor response to steroid therapy, which was not overcome by the high dose of steroid induced by the ITS therapy.

There are at least 3 requirements for successful IT treatment. Firstly, a secure and confirmable delivery method is necessary. For the agent to perfuse via the RW membrane, it is important to replace the air around the RW membrane [Nomura, 1984; Silverstein et al., 1997] with the solution containing the agent. Secondly, sequential or continuous administration of the drug might be desirable because it is expected to maintain the concentration of the drug in the target cells at a high level [Plontke et al., 2007]. Finally, it should be an easy and painless delivery method. The daily short-term IT DEX treatment using LAM seems to meet these 3 requirements.

In conclusion, daily short-term IT DEX treatment using LAM for ISSNHL patients without concurrent therapy is effective as an initial treatment as well as a salvage one and proved to be an alternative therapeutic option to high-dose systemic steroids. However, a prospective study is necessary to validate the conclusion.

References

Ahn JH, Yoo MH, Yoon TH, Chung JW: Can intratympanic dexamethasone added to systemic steroids improve hearing outcome in patients with sudden deafness? Laryngoscope 2008;118:279–282.

Battaglia A, Burchette R, Cueva R: Combination therapy (intratympanic dexamethasone + high-dose prednisone taper) for the treatment of idiopathic sudden sensorineural hearing loss. Otol Neurotol 2008;29:453–460.

Battista RA: Intratympanic dexamethasone for profound idiopathic sudden sensorineural hearing loss. Otolaryngol Head Neck Surg 2005;132:902–905.

Chandrasekhar SS: Intratympanic dexamethasone for sudden sensorineural hearing loss: clinical and laboratory evaluation. Otol Neurotol 2001;22:18–23.

Chandrasekhar SS, Rubinstein R, Kwartler J, Gatz M, Connelly PE, Huang E, Baredes S: Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents. Otolaryngol Head Neck Surg 2000;122:521–528.

Conlin AE, Parnes LS: Treatment of sudden sensorineural hearing loss. 1. A systemic review. Arch Otolaryngol 2007;133:573–581.

Dallan I, Bruschini P, Nacci A , Bruschini P, Traino C, Rognini F, Fattori B: Transtympanic steroids as a salvage therapy in sudden hearing loss: preliminary results. ORL J Otorhinolaryngol Relat Spec 2006;68:247–252.

Dallan I, De Vito A, Fattori B, Casani AP, Panicucci E, Berrettini S, Marchetti M, Nacci A: Intratympanic methylprednisolone in refractory sudden hearing loss: a 27-patient case series with univariate and multivariate analysis. Otol Neurotol 2010;31:25–30.

Gianoli GJ, Li J: Transtympanic steroids for treatment of sudden hearing loss. Otolaryngol Head Neck Surg 2001;125:142–146.

Han CS, Park JR, Boo SH, Jo JM, Park KW, Lee WY, Ahn JG, Kang MK, Park BG, Lee H: Clinical efficacy of initial intratympanic steroid treatment on sudden sensorineural hearing loss with diabetes. Otolaryngol Head Neck Surg 2009;141:572–578.

Haynes DS, O'Malley M, Cohen S, Watford K, Labadie RF: Intratympanic dexamethasone for sudden sensorineural hearing loss after failure of systemic therapy. Laryngoscope 2007;117:3–15.

Ho HG, Lin HC, Shu MT, Yang CC, Tsai HT: Effectiveness of intratympanic dexamethasone injection in sudden-deafness patients as salvage treatment. Laryngoscope 2004;114:1184–1189.

Hong SM, Park CH, Lee JH: Hearing outcomes of daily intratympanic dexamethasone alone as a primary treatment modality for ISSHL. Otolaryngol Head Neck Surg 2009;141:579–583.

Hu A, Parnes L: Intratympanic steroids for inner ear disorders: a review. Audiol Neurootol 2009;14:373–382.

Kakehata S, Futai K, Kuroda R, Shinkawa H: Office-based endoscopic procedure for diagnosis in conductive hearing loss cases utilizing OtoScan laser-assisted myringotomy. Laryngoscope 2004;114:1285–1289.

Kakehata S, Hirose Y, Kitani R, Futai K, Maruya S, Ishii K, Shinkawa H: Autologous serum eardrops therapy with a chitin membrane for closing tympanic membrane perforations. Otol Neurotol 2008;29:791–795.

Kakehata S, Saski A, Oji K, Futai K, Ota S, Makinae K, Shinkawa H: Comparison of intratympanic and intravenous dexamethasone treatment on sudden sensorineural hearing loss with diabetes. Otol Neurotol 2006;27:604–608.

Kanzaki J, Inoue Y, Ogawa K, Fukuda S, Fukushima K, Gyo K, Yanagihara N, Hoshino T, Ishitoya J, Toriyama M, Kitamura K, Murai K, Nakashima T, Niwa H, Nomura Y, Kobayashi H, Oda M, Okamoto M, Shitara T, Sakagami M, Tono T, Usami S: Effect of single-drug treatment on idiopathic sudden sensorineural hearing loss. Auris Nasus Larynx 2003;30:123–127.

Kara E, Cetik F, Tarkan O, Sürmelioğlu O: Modified intratympanic treatment for idiopathic sudden sensorineural hearing loss. Eur Arch Otorhinolaryngol 2010;267:701–707.

Kilic R, Safak MA, Oquz H, Kargin S, Demirci M, Samim E, Ozluoglu LN: Intratympanic methylprednisolone for sudden sensorineural hearing loss. Otol Neurotol 2007;28:312–316.

Kopke RD, Hoffer ME, Wester D, O'Leary MJ, Jackson RL: Targeted topical steroid therapy in sudden sensorineural hearing loss. Otol Neurotol 2001;22:475–479.

Lautermann J, Sudhoff H, Junker R: Transtympanic corticoid therapy for acute profound loss. Eur Arch Otorhinolaryngol 2005;262:587–591.

Niedermeyer HP, Zahneisen G, Luppa P, Busch R, Arnold W: Cortisol levels in the human perilymph after intravenous administration of prednisolone. Audiol Neurootol 2003;8:316–321.

Nomura Y: Otological significance of the round window. Adv Otorhinolaryngol 1984;33:66–72.

Parnes LS, Sun AH, Freeman DJ: Corticosteroid pharmacokinetics in the inner ear fluids: an animal study followed-up by clinical application. Laryngoscope 1999;109:1–17.

Plaza G, Herraiz C: Intratympanic steroids for treatment of sudden hearing loss after failure of intravenous therapy. Otolaryngol Head Neck Surg 2007;137:74–78.

Plontke SK, Löwenheim H, Mertens J, Engel C, Meisner C, Weidner A, Zimmermann R, Preyer S, Koitschev A, Zenner HP: Randomized, double-blind, placebo-controlled trial on the safety and efficacy of continuous intratympanic dexamethasone delivered via a round window catheter for severe to profound sudden idiopathic sensorineural hearing loss after failure of systemic therapy. Laryngoscope 2009;11:359–369.

Plontke SK, Siedow N, Wegener R, Zenner HP, Salt AN: Cochlear pharmacokinetics with local inner ear drug delivery using a three-dimensional finite-element computer model. Audiol Neurootol 2007;12:37–48.

Silverstein H, Rowan PT, Olds MJ, Rosenberg SI: Inner ear perfusion and the role of round window patency. Am J Otol 1997;18:586–589.

Van Wijck F, Staecker H, Lefebvre PP: Topical steroid therapy using the Silverstein Microwick in sudden sensorineural hearing loss after failure of conventional treatment. Acta Otolaryngol 2007;127:1012–1017.

Wilson WR, Byl FM, Laird N: The efficacy of steroids in the treatment of idiopathic sudden hearing loss: a double-blind clinical study. Arch Otolaryngol 1980;106:772–776.

耳鼻 56：145～150，2010

原 著

TRT 後の MCL・UCL 検査の変化

君付　隆・松本　希・柴田修明
玉江昭裕・大橋　充・梅野好啓
野口敦子・堀切一葉・小宗静男

Tinnitus Retraining Therapy（TRT）は、耳鳴を消失させるのではなく、順応により気にならなくさせることが目的の治療である。TRT により、耳鳴以外の外界からの音に対しても馴化を起こし、most comfortable loudness level（MCL）や uncomfortable loudness level（UCL）が変化するか否かを検討した。9名（男性4名、女性5名）に TRT を 4 − 22 カ月（平均 12 カ月）施行した。Tinnitus Handicap Inventory スコアは、59.7 ± 21.8 から 20.2 ± 10.1 へと改善したが、MCL・UCL 検査は両者とも明らかな変化を認めなかった。治療前の UCL が特に低値を示した症例においても、TRT による MCL・UCL の変化を認めなかった。

Key words：耳鳴、TRT、MCL・UCL 検査、順応

はじめに

TRT（Tinnitus Retraining Therapy）は、神経生理学的モデルに基づいた耳鳴の順応療法で、TCI（Tinnitus Control Instrument）による音響療法とカウンセリングから成り立っている[1]。TCI による音響治療は、雑音を与えることにより耳鳴と周辺雑音とのコントラストを減弱させ、大脳辺縁系での耳鳴に対する過敏性を減少させ、耳鳴に順応させる（habituation）治療である。これは耳鳴を消失させるのではなく、気にならなくさせることが目的である[2]。一方、most comfortable loudness level（MCL）検査・uncomfortable loudness level（UCL）検査は、通常外界からの純音に対して音が快適に聞こえる大きさ（MCL レベル）と、これ以上聞いてはいられない不快な大きさ（UCL レベル）を測定する検査で、補聴器のフィッティングの際に使われることが多い。また、内耳機能検査の一つとして、リクルートメント現象の有無の判定に使われることもある[3]。

TRT により耳鳴に順応し気にならなくなった場合、耳鳴以外の外界からの音に対しても馴化され、MCL・UCL レベルが変化する可能性がある。今回われわれは、耳鳴に対して TRT を行った前後で、MCL・UCL 検査に変化が起こるか否か検討した。

対象と方法

対象は九州大学病院耳鼻咽喉科外来を耳鳴で受診し、TRT を施行され治療前と治療後に MCL・UCL 検査を行った男性4名、女性5名の計9名である。年齢は 34 − 71 歳（平均 60.0 歳）、耳鳴耳の聴力レベルは 8.3 − 66.7 dB で、平均 29.7 dB であった。治療期間は 4 − 22 カ月（平均 12 カ月）であった。TRT の判定は、自覚症状については耳鳴の大きさと耳鳴の気になり方を標準耳鳴

九州大学医学部耳鼻咽喉科学教室
別刷請求：〒812-8582　福岡市東区馬出 3-1-1　九州大学医学部耳鼻咽喉科学教室　君付　隆

検査法1993（耳鳴研究会作成）の5段階評価に従って行った[4]。また、耳鳴の心理的苦痛度・生活障害度はNewmanのTinnitus Handicap Inventory（THI）scoreの日本語訳にて評価した[5]。MCL・UCL検査は250 Hz、1,000 Hz、4,000 Hzの連続音を用い、上昇法で測定した。測定順序は、左右のMCL検査を行った後に左右のUCL検査を行った。TRTのサウンドジェネレータは、シーメンスヒヤリングインストルメンツ社のTCIを使用した。統計処理はOrigin 6.1 Jにて解析し、t検定にて行った。

結　果

5段階評価では、耳鳴の大きさに関しては治療前の3.8 ± 0.8から治療後の2.7 ± 1.0へ、耳鳴の気になり方に関しては治療前の3.3 ± 1.0から治療後の2.1 ± 1.3へと変化した（図1）。両者とも統計学的に差は認めないものの（大きさ：$p = 0.06$、気になり方：$p = 0.09$）、減少傾向を認めた。THIスコアは、対象のすべての症例でスコアの減少を認め（図2 A）、平均で59.7 ± 21.8から20.2 ± 10.1へと有意差をもって減少した（図2 B）。障害度分類では、severe handicap（58 − 100）からmild handicap（18 − 36）まで改善したことになる。

MCL・UCL検査は、平均で右耳はMCLが48.3 dBから48.3 dB（250 Hz）、45.0 dBから46.6 dB（1,000 Hz）、47.2 dBから45.0 dB（4,000 Hz）、UCLが77.7 dBから75.0 dB（250 Hz）、78.3 dBから78.8 dB（1,000 Hz）、77.7 dBから75.5 dB（4,000 Hz）へと、MCL、UCLの両者とも変化を認めなかった（図3 A）。左耳もMCLが49.4 dBから48.8 dB（250 Hz）、45.5 dBから43.8 dB（1,000 Hz）、51.6 dBから50.0 dB（4,000 Hz）、UCLが77.7 dBから75.0 dB（250 Hz）、77.2 dBから77.2 dB（1,000 Hz）、81.6 dBから76.2 dB（4,000 Hz）へと、MCL、UCLの両者とも変化を認めなかった（図3 B）。

特に治療前のUCLが低値を示した症例について、TRTにてMCL・UCL値が変化するか否かをみた（図4）。症例は34歳、女性、聴力は左右とも正常、TRT前のUCLは45 − 65 dBと低値であった。6カ月間TRTを施行し、THIは38点から26点へとmoderate handicapからmild handicapまで改善した。しかし、MCL・UCL値は右250 Hzと右1,000 HzのMCLが上昇したものの、UCLは40 − 70 dBと明らかな変化を認めなかった。

考　察

TRTによりTHIスコアは平均で59.7から20.2へと減少した（図2 B）。20点以上の改善で臨床的に意義があるとされており[6]、耳鳴に対してTRTの有用性が確認された。しかし、MCL・UCL値についてはTRTによる変化を認めなかった（図3）。内因性の耳鳴と外界からの音に対する中枢の感受性には相違があることが考えられる。ラウドネス・バランス検査で、耳鳴のラウドネスは6 dB以内が70%、9 dB以内が84%とされており[7]、耳鳴の音量は外界音に換算すると非常に小さい。そのために、耳鳴に対してはTRTによる順応が可能であるが、MCL・UCL検査で与える大きな音に対しては、大脳辺縁系での過敏性を変化させることができず、MCL・UCL値が不変であった可能性が考えられる。また、耳鳴のマスキング検査（遮蔽検査）において、いかなるマスキング音でも耳鳴の完全なマスキングができない症例が存在するといわれている[8]。このことは耳鳴と外界からの音を感受する中枢の部位が異なることを意味し、そのために耳鳴において有効であったTRTがMCL・UCL検査に対しては影響を与えなかった可能性が考えられる。

耳鳴患者の平均UCLは、TRT前で77.2 dB − 81.6 dB、TRT後で75.0 dB − 78.8 dBであった。通常UCLは90 dB前後とされるが[9]、今回の症例では低値となっている。耳鳴患者の20 − 45 %程度は聴覚過敏が合併するといわれており[10]、UCLの低下は聴覚過敏の側面を反映しているの

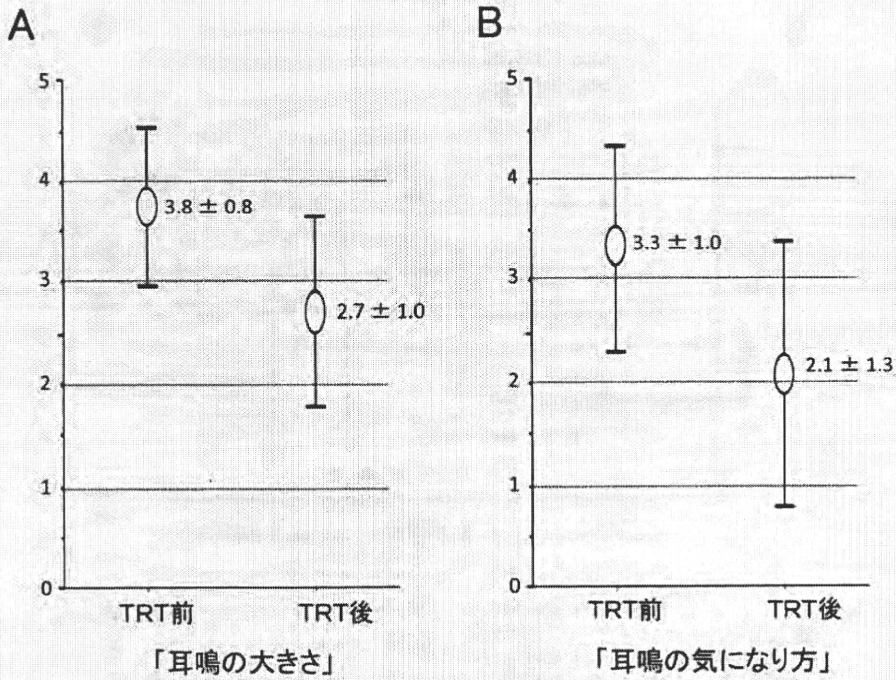


図1　TRT による5段階評価の変化

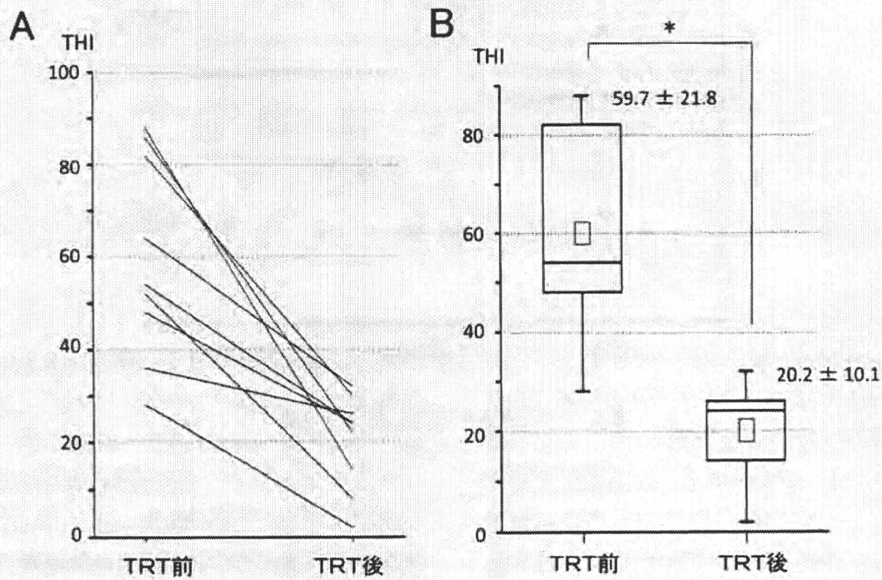


図2　TRT による THI スコアの変化
A：全症例の THI の変化（n = 9）　B：A のボックスチャート。最大値；75 パーセンタイル、中央値：25 パーセンタイル、最小値を示す。□は平均値、数字は平均±標準偏差を示す。

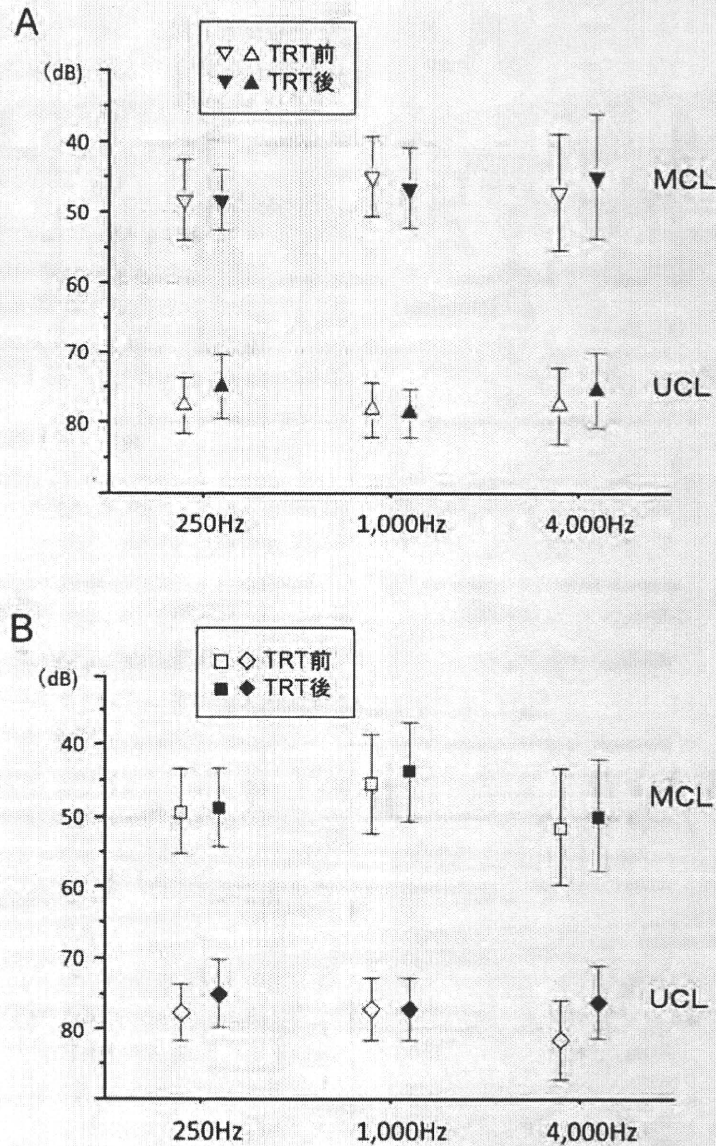


図3　TRT 前後の MCL・UCL 平均値
A：右耳　B：左耳

かもしれない。UCL 値の低下が著しい症例について、TRT により外界からの音に対しても順応し UCL 値が上昇することも考えられたが、実際は変化しなかった（図4）。UCL 低値は、内耳障害におけるリクルートメント現象を反映している[3]。リクルートメント現象の発生源についてはいまだ定説はないが、Tonndorf[11] は、有毛細胞の感覚毛と蓋膜の関係を唱えている。すなわち、感覚毛と蓋膜が離れていると低音圧刺激では刺激が伝わらず反応閾値が上昇するが、強音圧刺激に

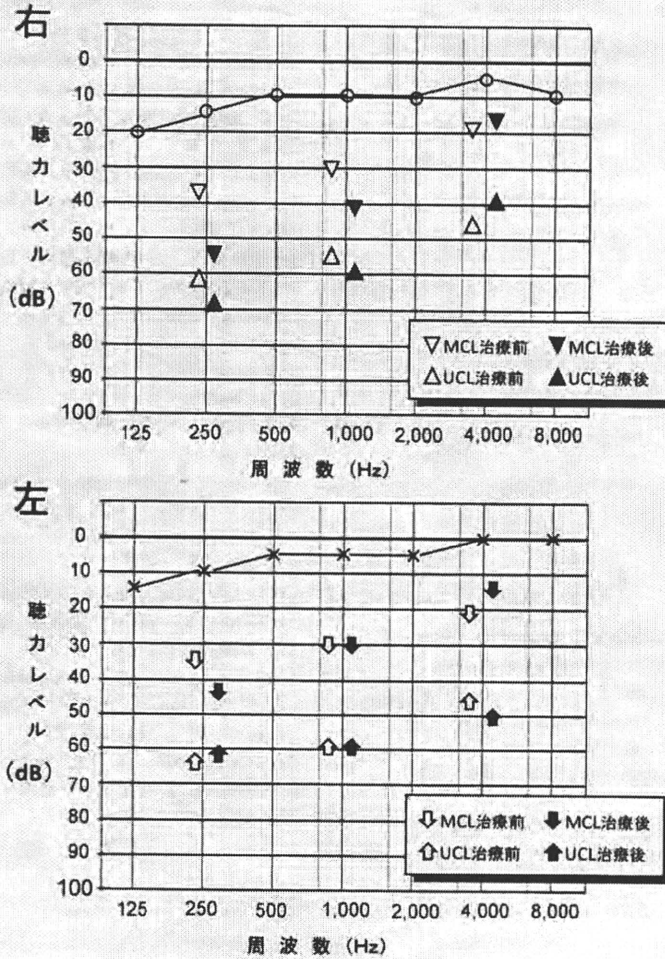


図4 症例の MCL・UCL 変化

おいては感覚毛が蓋膜に接し、出力曲線の急峻なのびを示し、リクルートメント現象として観察されるという説である。また Center-clipping という内有毛細胞に特異な現象についても言及している。いずれにしても、低値を示す UCL は内耳に起因しているため、TRT で大脳辺縁系での過敏性を変化させても、UCL 自体には影響を与えなかったと考えられる。

耳鳴については不明の点が多く、原因についても諸説があるが、近年、内有毛細胞から神経伝達物質を直接受けるラセン神経節細胞の受容体の変化が、耳鳴の原因の一つであると報告された[12)-14)]。内有毛細胞からラセン神経節細胞への神経伝達物質はグルタミン酸であり、ラセン神経節細胞には、グルタミン酸レセプターのサブタイプである AMPA（α-アミノ-3-ヒドロキシ-5-メソオキサゾール-4-プロピオン酸）レセプターが存在する。しかし、サリチル酸による耳鳴を発症させた場合、動物モデルでも[12)]、in vitro 研究でも[13)] AMPA レセプターのほかに NMDA（N-メ

チル-D-アスパラギン酸）レセプターも活性化されることが証明された。この新たに活性化されたNMDAレセプターを介する神経活動が、耳鳴の原因であるという説である。NMDAレセプターの阻害薬が耳鳴を軽減するとの臨床報告もある[14]。すなわち、通常の外界からの音を感知する場合はAMPAレセプターが働き、耳鳴の場合はNMDAレセプターが働くということになる。この説に従うと、今回の報告のMCL・UCL検査ではAMPAレセプター、耳鳴ではNMDAレセプターが関与することになり、NMDA関与の耳鳴をTRTで順応させても、AMPA関与のMCL・UCL検査はTRTで影響を受けなかったと考えられる。

文 献

1) Jastreboff PJ : Phantom auditory perception (tinnitus) − Mechanisms of generation and perception −. Neurosci Res **8** : 221-254, 1990.
2) 関谷芳正他：耳鳴に対する新しい治療法・TRT（療法）. 耳鼻臨床 **95** : 639-646, 2002.
3) 君付 隆他：MCL・UCL検査の判定基準. 耳鼻 **54** : 140-145, 2008.
4) 耳鳴研究会：標準耳鳴検査法1993. 耳鳴の検査. 立木孝・曽田豊二編, 111-116頁, 金原出版, 東京, 1999.
5) 高橋真理子・村上信五：耳鳴治療のための耳鳴評価法. ENTONI **49** : 34-39, 2000.
6) Newman CW et al : Psychometric adequancy of the Tinnitus Handicap Inventory (THI) for evaluating treatment outcome. J Am Acad Audiol **9** : 153-160, 1998.
7) Vernon JA and Meikle MB : Tinnitus clinical measurement. Otolaryngol Clin North Am **36** : 293-305, 2003.
8) Feldmann H : Homolateral and contralateral masking of tinnitus by noise-bands and pure tones. Audiology **10** : 138-144, 1971.
9) Sammeth CA et al : Variability of most comfortable and uncomfortable loudness levels to speech stimuli in the hearing impaired. Ear Hear **10** : 94-100, 1989.
10) 新谷朋子他：耳鳴と聴覚過敏. JOHNS **23** : 15-18, 2007.
11) Tonndorf J : Stereociliary dysfunction, a cause of sensory hearing loss, recruitment, poor speech discrimination and tinnitus. Acta Otolaryngol **91** : 469-479, 1981.
12) Guitton MJ et al : Salicylate induced tinnitus through activation of cochlear NMDA receptors. J Neurosci **23** : 3944-3952, 2003.
13) Ruel J et al : Salicylate enables cochlear arachidonic-acid-sensitive NMDA receptor responses. J Neurosci **28** : 7313-7323, 2008.
14) Figueiredo RR et al : Tinnitus treatment with memantine. Otolaryngol Head Neck Surg **138** : 492-496, 2008.

（受付 2010年4月26日）

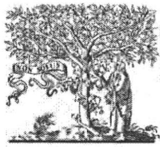
Changes in the MCL/UCL test for patients undergoing Tinnitus retraining therapy (TRT)

Takashi KIMITSUKI, Nozomu MATSUMOTO, Shumei SHIBATA, Akihiro TAMAE, Mitsuru OHASHI, Yoshihiro UMENO, Atsuko NOGUCHI, Kazuha HORIKIRI and Shizuo KOMUNE

Department of Otorhinolaryngology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan

Tinnitus retraining therapy (TRT) has become a popular treatment for the treatment of patients with tinnitus. This method involves the habituation of tinnitus perception using a low level sound generator and directive counseling. In this paper, we examined whether the most comfortable loudness level (MCL) and uncomfortable loudness level (UCL) changed after undergoing TRT. Nine patients (four males, five females) underwent TRT for 4-22 months (for an average of 12 months). The tinnitus handicap inventory (THI) score improved from 59.7 to 20.2, but neither the MCL nor the UCL showed any changes after TRT. In one patient where the UCL was particularly low prior to treatment, no changes were observed in either MCL or UCL after TRT.

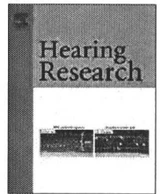
Hearing Research 261 (2010) 57–62



Contents lists available at ScienceDirect

Hearing Research

journal homepage: www.elsevier.com/locate/heares



Research paper

Property of $I_{K,n}$ in inner hair cells isolated from guinea-pig cochlea

Takashi Kimitsuki *, Noritaka Komune, Teppei Noda, Kazutaka Takaiwa, Mitsuru Ohashi, Shizuo Komune

Department of Otolaryngology, Graduate School of Medical Sciences, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higasi-Ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 12 September 2009
Received in revised form 4 January 2010
Accepted 5 January 2010
Available online 12 January 2010

Keywords:
Cochlea
Inner hair cell
Potassium currents
KCNQ-channel
Linopirdine
XE991

ABSTRACT

One of the potassium currents, $I_{K,n}$, is already activated at the resting potential of the cell and thus determines the membrane potential. KCNQ4 channel has been identified as the molecular correlate of $I_{K,n}$. In the present study, we measured $I_{K,n}$ in acutely isolated IHCs of guinea-pig cochlea using the whole-cell voltage-clamp techniques, and investigated the properties of the currents. $I_{K,n}$ was 70% activated around the resting potential of −60 mV and deactivated on hyperpolarization. $I_{K,n}$ was blocked by the KCNQ-channel blockers, linopirdine (100 μM) and XE991 (10 μM), but was insensitive to both $I_{K,f}$ blocker, tetraethylammonium (TEA), and $I_{K,s}$ blocker, 4-aminopyridine (4-AP). There was no significant difference in the size of $I_{K,n}$ between the apical and basal turn IHCs.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

In the mammalian cochlea, there are two types of hair cells that subserve distinct functions and receive characteristic patterns of innervations. Inner hair cells (IHCs) receive nearly all the afferent innervations and are primary acoustic transducers. The three IHC potassium currents are distinguishable by their pharmacology and their activation kinetics (Kros and Crawford, 1990; Marcotti et al., 2003). The fast activating current, $I_{K,f}$, is blocked by TEA but resistant to 4-AP. $I_{K,s}$ is activated more slowly on depolarization and is blocked by 4-AP but not by TEA. Another potassium current, $I_{K,n}$, is already activated at the resting potential of the cell and thus determines the membrane potential and membrane constant (Housley and Ashmore, 1992; Nakagawa et al., 1994; Marcotti and Kros, 1999). Initially $I_{K,n}$ was identified in outer hair cells (OHCs) (Housley and Ashmore, 1992). Although there is no detectable expression of KCNQ4, which is thought to constitute the major conductance $I_{K,n}$, in IHCs (Beisel et al., 2000; Kharkovets et al., 2000), a recent article suggested that KCNQ4 is expressed in IHCs (Oliver et al., 2003). This current has recently been implicated in developmental changes in IHCs during the postnatal days just preceding functional maturation of hearing in mice (Kros et al., 1998; Marcotti et al., 2003).

$I_{K,n}$ in IHCs has been identified and characterized in mice previously (Oliver et al., 2003; Marcotti et al., 2003), however, it is important to confirm this current in guinea-pig, which is a commonly used model. In the present study, we isolated the IHCs from mature guinea-pig cochlea and investigated the properties of $I_{K,n}$ such as the K blocker effect and the activation properties. Tonotopic differences of $I_{K,n}$ in the cochlear turn were also investigated.

2. Material and methods

2.1. Preparation of isolated IHCs

An adult albino guinea-pig (200–350 g, 3–6 weeks) was killed by rapid cervical dislocation, both bullae were removed and the cochlea exposed. The cochlea, fused to the bulla, was placed in a Ca^{2+}-free external solution (mM: 142 NaCl, 4 KCl, 3 $MgCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH). The otic capsule was opened, allowing removal of the organ of Corti attached to the modiolus. IHCs were isolated by micro-dissecting a selected turn of the organ of Corti; from turn 1–2 (basal portion) and turn 4 (apical portion). The organ of Corti was treated with trypsin (0.5 mg/ml, T-4665, Sigma) for 12 min, and gentle mechanical trituration was carried out. Trypsin was rinsed from the specimen by superfusing with a standard external solution (mM: 142 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH) for at least 10 min before starting any experiments. The most important landmarks for identifying IHCs are a tight neck and angle between the cuticular plate and the axis of the cell as

Abbreviations: IHC, inner hair cell; OHC, outer hair cell; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; CAP, compound action potential

* Corresponding author. Tel.: +81 92 642 5668; fax: +81 92 642 5685.
E-mail address: kimitaka@qent.med.kyushu-u.ac.jp (T. Kimitsuki).

0378-5955/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.heares.2010.01.002

described previously (He et al., 2000; Yang et al., 2002; Kimitsuki et al., 2009).

2.2. Recording procedures

Membrane currents were measured by conventional whole-cell voltage-clamp recordings using an EPC-10 (HEKA, Lambrecht, Germany). Data acquisition was controlled by the software PatchMaster (HEKA, Lambrecht, Germany). Recording electrodes were pulled on a two-stage vertical puller (PP830 Narishige, Tokyo, Japan) using 1.2 mm outside diameter borosilicate glass (GC-1.2, Narishige, Tokyo) filled with an internal solution (mM: 144 KCl; 2 $MgCl_2$; 1 NaH_2PO_4; 8 Na_2HPO_4; 2 ATP; 3 D-glucose; 0.5 EGTA; adjusted to pH 7.4 with KOH.). Pipettes showed a resistance of 4–8 MΩ in the bath and were coated with ski wax (Tour-DIA, DIAWax, Otaru, Japan) to minimize capacitance. The cell's capacitance was 9.6 ± 3.0 pF (mean ± SD) and the series resistance was 16.4 ± 6.1 MΩ (n = 16). Tetraethylammonium (TEA, T-2265, Sigma), 4-aminopyridine (4-AP, A-0152, Sigma), Linopirdine (L-134, Sigma), XE991 (No. 2000, Tocris), replacing an equivalent amount of NaCl in the standard external solution, was applied under pressure (Pressure micro-injector: PMI-200, Dagan, Minneapolis) using pipettes with a tip diameter of 2–4 μm positioned around 50 μm from the IHCs. Cells were continuously perfused with external saline and all experiments were performed at room temperature (20–25 °C).

2.3. Animal care

The experimental design was reviewed and approved (Accession No. A19-104-0) by the Animal Care and Use Committee, Kyushu University. All procedures were conducted in accordance with the Guidelines for Animal Care and Use Committee, Kyushu University.

3. Results

3.1. $I_{K,n}$ activated at resting potentials

Currents in response to hyperpolarizing and depolarizing voltage steps from a holding potential of −60 mV were recorded from IHCs. Typical current records are shown in Fig. 1A. IHCs had outwardly rectifying currents ($I_{K,f}$) in response to depolarizing voltage pulses, with only a slight inward current when hyperpolarized. Fig. 1B demonstrated the inward components by enlarging the scale. Sizeable currents were detected as decaying inward currents that commenced instantaneously when the membrane was stepped below −90 mV. At hyperpolarized potentials the decay time of the transient, indicating the channel deactivation, varied considerably depending on membrane potentials. These deactivating inward currents corresponded to $I_{K,n}$ previously found in OHCs (Housley and Ashmore, 1992; Nakagawa et al., 1994; Marcotti and Kros, 1999) and immature IHCs (Kros et al., 1998; Marcotti et al., 2003) already activated at resting potentials.

3.2. voltage-dependent activation of $I_{K,n}$

The voltage-dependent activation of $I_{K,n}$ was examined by analyzing the peak of the tail currents at a fixed membrane potential of −160 mV, following depolarizing and hyperpolarizing voltage steps. In most cases, the tail currents observed from large hyperpolarizing steps (−140 to −160 mV) overlapped, suggesting that $I_{K,n}$ was fully turned off at these negative potentials. Conversely, there was overlap of the traces when the pre-pulse was greater than −20 mV, which is interpreted as complete activation of the current. Fig. 2 shows an activation curve derived for $I_{K,n}$, fitted by first-order Boltzmann equation:

$$I/I_{max} = 1/[1 + \exp((V_{half} - V)/S)]$$

where V_{half} is the potential of half-maximal activation, V is the membrane potential of the preceding voltage step and S describes the voltage sensitivity of activation. Fitting was performed by using the average values (filled squares) at various membrane potentials in 10 cells. V_{half} and S were −84.5 mV and 25.3 mV, respectively.

3.3. K channel blocker effect to $I_{K,n}$

The fast activating current, $I_{K,f}$, is blocked by TEA and slow activating current, $I_{K,s}$, is blocked by 4-AP. The effects of 25 mM TEA (Fig. 3A) and 30 mM 4-AP (Fig. 3B) were investigated. Both K channel blockers reduced the amplitude of outward-going currents (insets in Fig. 3A showed the TEA block onto the fast activating $I_{K,f}$) but did not affect the inward currents, suggesting that $I_{K,n}$ is insensitive to TEA and 4-AP. KCNQ4 channel has been identified as the molecular correlate of the $I_{K,n}$ in OHCs (Marcotti and Kros, 1999)

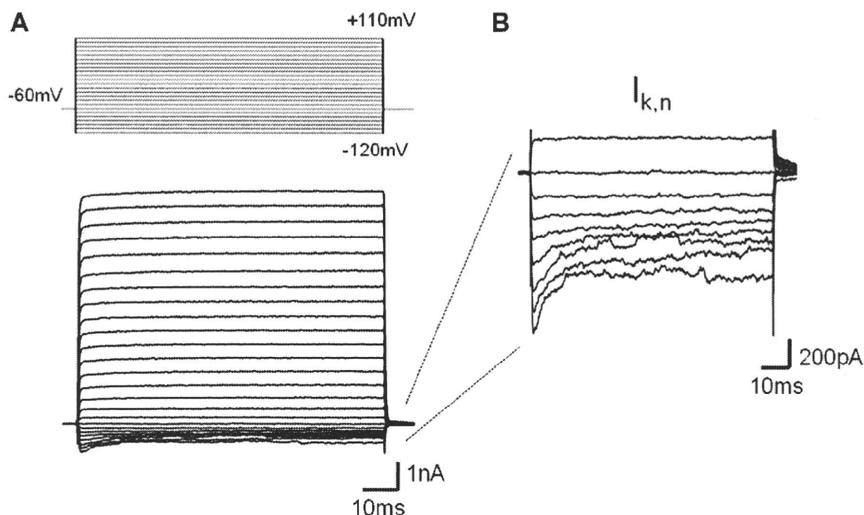


Fig. 1. $I_{K,n}$ activated at resting potentials. Currents in response to hyperpolarizing and depolarizing voltage steps from a holding potential of −60 mV (A, lower panel). Voltage protocol (A, upper panel). The inward component of the currents by enlarging the scale (B).

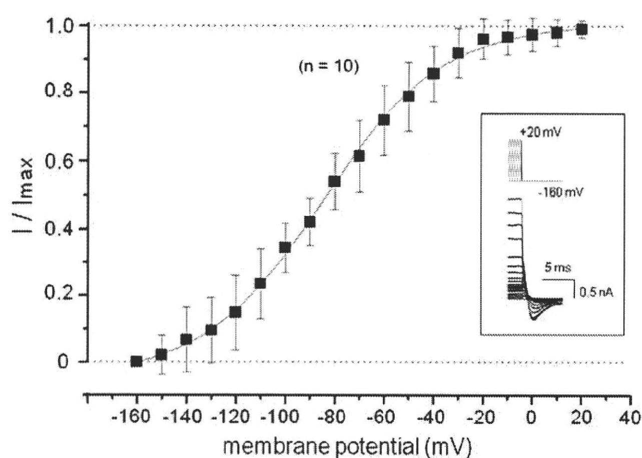


Fig. 2. Voltage-dependent activation of $I_{K,n}$. The voltage-dependent activation was evaluated by analyzing the tail currents at a fixed membrane potential of −160 mV, following depolarizing and hyperpolarizing voltage steps (inset). An activation curve derived from $I_{K,n}$ fitted by first-order Boltzmann equation: $I/I_{max} = 1/[1 + \exp((V_{half} - V)/S)]$. V_{half} and S were −84.5 mV and 25.3 mV, respectively.

and IHCs (Oliver et al., 2003), so we used the KCNQ-channel blocker linopirdine and XE991 to examine whether they block the currents. Inward currents were obviously blocked by 100 μM linopirdine (Fig. 4A) and 10 μM XE991 (Fig. 4B). In contrast, outward currents were not changed by either linopirdine or XE991, suggesting that only inward currents consist of $I_{K,n}$. In linopirdine solutions, four out of five cells showed the inward current depression, and in XE991 solutions, three out of four cells showed the inward current depression.

3.4. Amplitude of $I_{K,n}$ in apical and basal turn IHCs

A comparison of the amplitude of $I_{K,n}$ recorded from apical and basal turn IHCs is shown in Fig. 5. The amplitudes of inward currents at −130 mV were measured from seven apical cells and five basal cells, and shown as a boxplot. Minimum value (×), 25th percentile line, median line, 75th percentile line and maximum value (×) were shown. Mean (open square) ± standard deviation were 377 ± 192 pA and 338 ± 113 pA in apical and basal IHCs, respectively, showing that there was no difference between apical and basal turn IHCs in $I_{K,n}$ amplitude ($P = 0.65$ in analysis of variance: ANOVA). The cell capacitances were 9.3 ± 3.0 pF and 9.0 ± 3.5 pF in apical and basal IHCs, respectively, suggesting that current density is not different for the two tonotopic locations. Maximum currents from tails (fixed at −160 mV) were compared to avoid the influence of open probability. Mean ± standard deviation were 146 ± 62 pA ($n = 4$) and 154 ± 66 pA ($n = 3$) in apical and basal turn, respectively, suggesting that the voltage dependence of this current is independent of tonotopic location.

4. Discussion

Using whole-cell voltage-clamp recordings, we studied the $I_{K,n}$ in acutely isolate IHCs of guinea-pig cochlea. $I_{K,n}$ was 70% activated at around the resting potential of −60 mV and deactivated on hyperpolarization (Fig. 2). $I_{K,n}$ was blocked by the KCNQ-channel blockers linopirdine and XE991 but was insensitive to TEA and 4-AP (Figs. 3 and 4). There was no significant difference in the size of $I_{K,n}$ between the apical turn and basal turn.

Recently, mutations in the KCNQ4 K^+ channel gene have been shown to underlie the progressive autosomal dominant hearing loss classified as DFNA2 (Kubisch et al., 1999). KCNQ4 is expressed in the basolateral membrane of cochlear outer hair cells (OHCs) and is thought to constitute the major OHC K^+ conductance $I_{K,n}$ (Housley and Ashmore, 1992; Marcotti and Kros, 1999; Kharkovets et al., 2000). To date, expression of KCNQ4 has been thought to be restricted to cochlear OHCs, vestibular hair cells, and central auditory neurons (Kubisch et al., 1999; Kharkovets et al., 2000). However, recent reports demonstrated the expression of KCNQ4 in IHCs of mice using immunofluorescence (Oliver et al., 2003) or *in situ* hybridization and RT-PCR analysis (Beisel et al., 2000). The

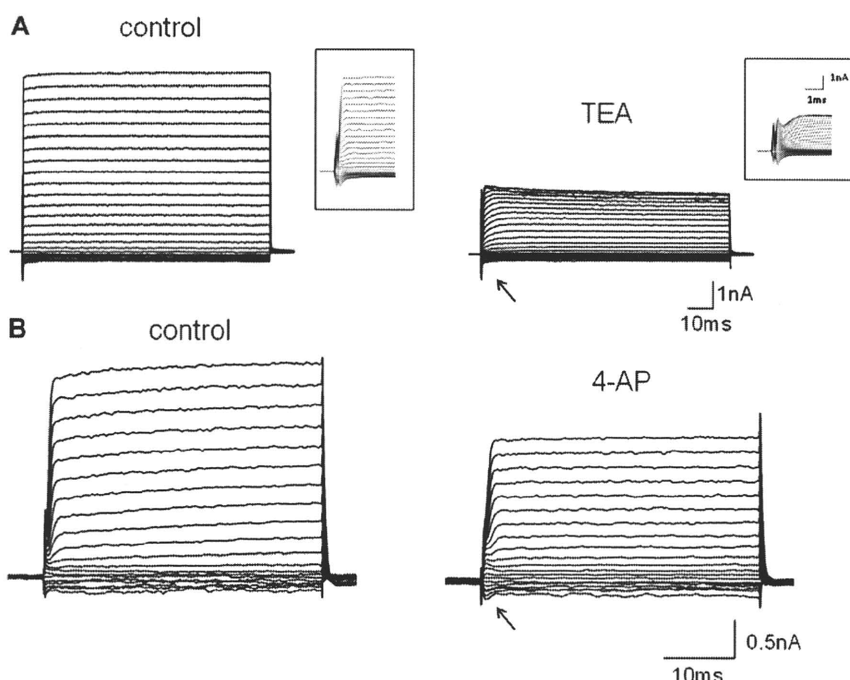


Fig. 3. TEA and 4-AP effects on $I_{K,n}$. The outward currents were blocked by 25 mM TEA (A) and 30 mM 4-AP (B). Inward currents were insensitive to both TEA and 4-AP (arrows). *Insets* in Fig. 3A showed the expanded time scale in the activating phase of the currents. TEA blocked the fast activating outward $I_{K,f}$.

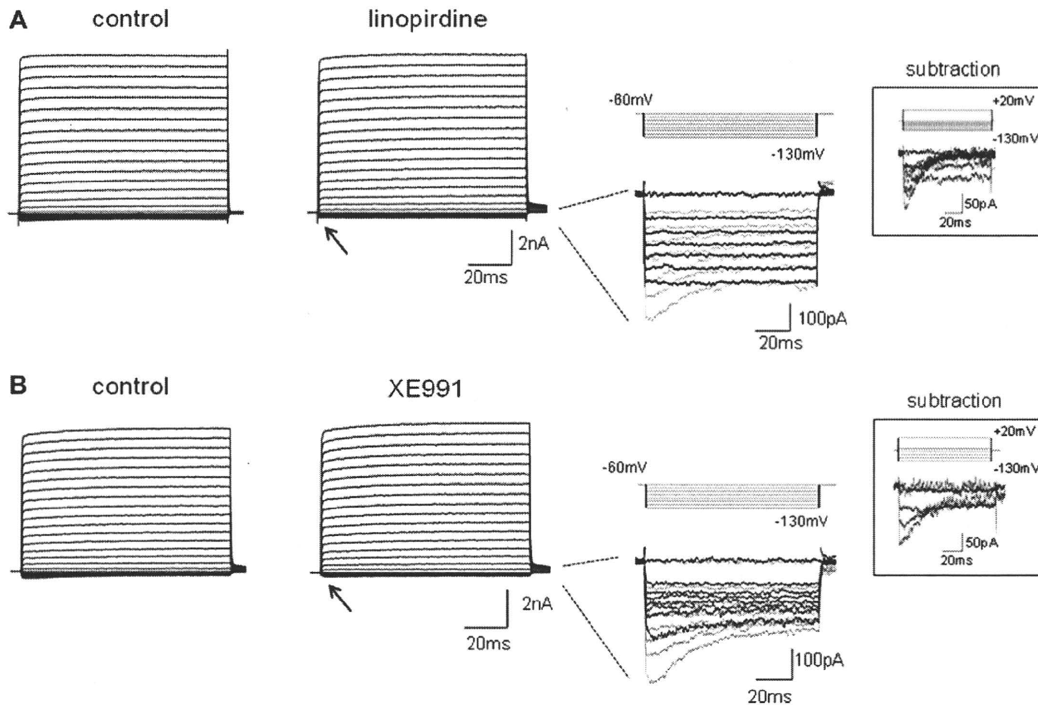


Fig. 4. Linopirdine and XE991 effects to $I_{K,n}$. Inward currents were blocked by the KCNQ-channel blocker 100 μM linopirdine (A) and 10 μM XE991 (B). Inward currents are shown by enlarging scale (right panel). Gray traces indicate control currents and black traces indicate $I_{K,n}$ with linopirdine or XE991. Insets showed the linopirdine- or XE991-sensitive currents by subtracting blocker traces from controls.

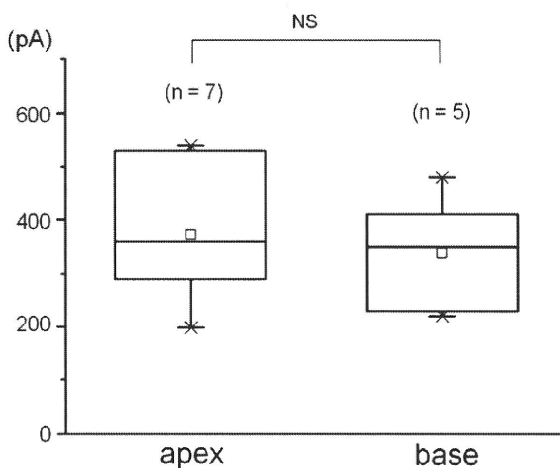


Fig. 5. Comparison between the amplitudes of $I_{K,n}$ in apical and basal turn IHCs. The amplitudes of inward currents at −130 mV were compared. Minimum value (×), 25th percentile line, median line, 75th percentile line and maximum value (×) were shown. Open squares indicate the mean values in the apical (377 pA) and basal turn (338 pA).

size of $I_{K,n}$ of IHCs was 45% of that of OHCs on comparison of 264 pA in IHCs (at a membrane potential of −124 mV: Marcotti et al., 2003) and 584 pA in OHCs (Marcotti and Kros, 1999). The amplitude of $I_{K,n}$ at −130 mV was 358 pA (Fig. 5, $n = 12$) in the present data, which is slightly larger than that in the previous report. The size of $I_{K,n}$ of IHCs was 61% of that of OHC, that is matching the respective sizes of the transducer conductance of IHCs and OHCs (Kros et al., 1992).

In the present study, trypsin was used and mechanical trituration was carried out to isolate the IHCs. Trypsin diminished the inactivation of BK channel (Kimitsuki et al., 2005) by attacking the N-terminal cytosolic hydrophobic peptide segments of auxiliary β subunit (Zhang et al., 2009). However, $I_{K,n}$ from the isolated IHCs, which is similar to that from the semi-intact preparation (Marcotti et al., 2003; Oliver et al., 2003), suggested that trypsin and trituration is inconsequential.

The first sign of $I_{K,n}$ was seen at postnatal day 12 (P12) in mouse IHCs (Marcotti et al., 2003), when the one-to-one axosomatic configuration between afferent fibers and IHCs found in mature synapses is established and the hearing onset occurs. The amplitude of $I_{K,n}$ increased and reached the maximum level at around P20. In the immunofluorescence study, KCNQ4 expression was observed initially around embryonic day 18.5 (E18.5) in the basal turn and proceeded longitudinally toward the apex (Beisel et al., 2000). At P8, the basal hook region showed the adult expression pattern and developmental upregulation had reached the apical turn. At P21, all hair cells, except those in the apical tip, had acquired the adult pattern. In the present study, $I_{K,n}$ was recorded from adult guinea-pigs, so the amplitude was slightly larger compared to the previous report (Marcotti et al., 2003).

There was a significant difference in the size of $I_{K,n}$ between the apical and basal turn IHCs in mice, showing larger $I_{K,n}$ in apical IHCs than that in basal IHCs (Marcotti et al., 2003). However, the resting membrane potential, that is mainly established by $I_{K,n}$, was similar in apical and basal IHCs. In contrast, the highest expression levels of KCNQ4 were found in the basal turn (Beisel et al., 2000), those findings contradicted the electrophysiological findings (Marcotti et al., 2003). Beisel et al. observed the longitudinal expression in the KCNQ4 protein by immunofluorescence technique, but they could not clarify the function of KCNQ4 K^+ channel. In the present study, there were no significant differences in $I_{K,n}$ amplitude between apical and basal turn IHCs (Fig. 5). This discrepancy might be due to the difference of species.

KCNQ K^+-channel blocker linopirdine has been a useful tool to define heterologous and native KCNQ currents (Wang et al., 1998; Kubisch et al., 1999; Lerche et al., 2000; Schroeder et al., 2000). The inward currents were insensitive to TEA and 4-AP but obviously blocked by the linopirdine (Fig. 4A) and XE991

(Fig. 4B). In mouse IHCs, blockage of $I_{K,n}$ by linopirdine was dose-dependent and slowly reversible with an IC_{50} of 0.58 μM (Oliver et al., 2003) or 0.56 μM (Marcotti et al., 2003). The concentration used in the present study was sufficient to block $I_{K,n}$ completely. *In vivo* study for guinea-pig, linopirdine significantly increased the threshold of the compound action potential (CAP) with an IC_{50} of 101 μM (Nouvian et al., 2003). They indicated that there was no recovery of CAP threshold after rinsing the cochlea with control artificial perilymph solutions. The blocking effect of another KCNQ-channel blocker XE991 for $I_{K,n}$ was described in mouse IHCs (Oliver et al., 2003). Sensitivity to XE991 was even higher than that to linopirdine because 100 nM blocked 77% of the current, and XE991-induced inhibition was irreversible contrary to the effect of linopirdine. XE991 block for KCNQ-channel was clearly demonstrated in OHCs in wild mouse ($Kcnq4^{+/+}$) but XE991-sensitive component was significantly reduced in $Kcnq4^{+/-}$ mice and was completely abolished in $Kcnq4^{-/-}$ mice (Kharkovets et al., 2006).

$I_{K,n}$, although small compared to the other outward currents $I_{K,f}$ and $I_{K,s}$, plays an important role close to the resting membrane potential because it showed around 70% activity in the present study (Fig. 2). $I_{K,n}$ provides a large K^+ conductance at the resting potential, and thus determines the membrane potential and membrane time constant (Housley and Ashmore, 1992; Marcotti and Kros, 1999). Although the $I_{K,f}$ blocker TEA did not change the resting potential (Oliver et al., 2003; Kros and Crawford, 1990) and the $I_{K,s}$ blocker 4-AP slightly shifted the resting potentials (Kros and Crawford, 1990), the $I_{K,n}$ blocker linopirdine markedly shifted the resting potentials toward a depolarization direction (Marcotti et al., 2003). Thus, $I_{K,n}$ sets the resting membrane potential and consequently maintains the intracellular Ca^{2+} at a low level by keeping the Ca^{2+} channels on closed state (Oliver et al., 2003). Another role of $I_{K,n}$ is the efflux route of K^+ ions to the perilymphatic space and prevention of K^+ accumulation in the cells. In OHCs, $I_{K,n}$ channels are located at the basolateral membrane facing the perilymphatic space (Nakagawa et al., 1994). Loss of this conductance in KCNQ4/DFNA2 patients is considered to impair K^+ efflux from OHCs, leading to degeneration of OHCs (Jentsch, 2000; Kharkovets et al., 2000). $I_{K,n}$ may also be important in IHCs to provide an exit route for K^+ ions entering through the mechano-electrical transducer channels.

KCNQ4 channels have been associated with the nonsyndromic dominant deafness DFNA2 (Kubisch et al., 1999; Talebizadeh et al., 1999; Van Hauwe et al., 2000; Van Camp et al., 2002). DFNA2 is characterized by a slowly progressive hearing loss that develops from high to low frequencies and finally leads to severe deafness (Marres et al., 1997; De Leenheer et al., 2002). In knockout and knock-in mouse, the hearing loss in DFNA2 is predominantly caused by the slow degeneration of OHCs due to chronic depolarization (Kharkovets et al., 2006), but complete loss of OHCs will ultimately reduce the hearing threshold by 40–50 dB (Ryan and Dallos, 1975). Although the loss of OHCs is compatible with DFNA2 hearing loss in its early stages, profound hearing loss in DFNA2 at the later age is insufficiently explained by nonfunctional OHCs. There might be an additional impairment of IHCs at the age when presbyacusis sets in. Destabilization of the resting potential and increase intracellular Ca^{2+}, which may be caused by impaired KCNQ4 function in IHCs, may promote the progressive hearing loss observed in DFNA2 patients. KCNQ4 is also expressed in spiral ganglion neurons (Beisel et al., 2005), and the nuclei of many neurons in the central auditory pathway, e.g., in the cochlear nuclei, nuclei of the lateral lemniscus, and the inferior colliculus (Kharkovets et al., 2000). The profound deafness in DFNA2 patients may be due to the later dysfunction of the KCNQ4 channels expressed in the nuclei and tracts of the central auditory pathway.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research 9591978, 21592160 from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Research Fund of Institute of Kampo Medicine (Japan).

References

Beisel, K.W., Nelson, N.C., Delimont, D.C., Fritzsch, B., 2000. Longitudinal gradients of KCNQ4 expression in spinal ganglion and cochlear hair cells correlate with progressive hearing loss in DFNA2. Brain Res. Mol. Brain Res. 82, 137–149.

Beisel, K.W., Rocha-Sanchez, S.M., Morris, K.A., Nie, L., Feng, F., Kachar, B., Yamoah, E.N., Fritzsch, B., 2005. Differential expression of KCNQ4 in inner hair cells and sensory neurons is the basis of progressive high-frequency hearing loss. J. Neurosci. 25, 9285–9293.

De Leenheer, E.M., Huygen, P.L., Coucke, P.J., Admiraal, R.J., van Camp, G., Cremers, C.W., 2002. Longitudinal and cross-sectional phenotype analysis in a new, large Dutch DFNA2/KCNQ4 family. Ann. Otol. Rhinol. Laryngol. 111, 267–274.

He, D.Z.Z., Zheng, J., Edge, R., Dallos, P., 2000. Isolation of cochlear inner hair cells. Hear. Res. 145, 156–160.

Housley, G.D., Ashmore, J.F., 1992. Ionic currents of outer hair cells isolated from guinea pig cochlea. J. Physiol. 448, 73–98.

Jentsch, T.J., 2000. Neuronal KCNQ potassium channels: physiology and role in disease. Nat. Rev. Neurosci. 1, 21–30.

Kharkovets, T., Hardelin, J.P., Safieldddine, S., Schweizer, M., El-Amraoui, A., Petit, C., Jentsch, T.J., 2000. KCNQ4, a K^+ channel mutated in a form of dominant deafness, is expressed in the inner ear and the central auditory pathway. Proc. Natl. Acad. Sci. USA 97, 4333–4338.

Kharkovets, T., Dedek, K., Maier, H., Schweizer, M., Khimich, D., Nouvian, R., Vardanyan, V., Leuwer, R., Moser, T., Jentsch, T.J., 2006. Mice with altered KCNQ4 K+ channels implicate sensory outer hair cells in human progressive deafness. EMBO J. 25, 642–652.

Kimitsuki, T., Ohashi, M., Wada, Y., Fukudome, S., Komune, S., 2005. Dissociation enzyme effects on the potassium currents of inner hair cells isolated from guinea-pig cochlea. Hear. Res. 199, 135–139.

Kimitsuki, T., Kakazu, Y., Matsumoto, N., Noda, T., Komune, N., Komune, S., 2009. Salicylate-induced morphological changes of isolated inner hair cells and outer hair cells from guinea-pig cochlea. Auris Nasus Larynx 36, 152–156.

Kros, C.J., Crawford, A.C., 1990. Potassium currents in inner hair cells isolated from the guinea-pig cochlea. J. Physiol. 421, 263–291.

Kros, C.J., Rusch, A., Richardson, G.P., 1992. Mechano-electrical transducer currents in hair cells of the cultured neonatal mouse cochlea. Proc. R. Soc. Lond. 249, 185–193.

Kros, C.J., Ruppersberg, J.P., Rusch, A., 1998. Expression of a potassium current in inner hair cells during development of hearing in mice. Nature 394, 281–284.

Kubisch, C., Schroeder, B.C., Friedrich, T., Lutjohann, B., El-Amraoui, A., Marlin, S., Petit, C., Jentsch, T.J., 1999. KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 96, 437–446.

Lerche, C., Scherer, C.R., Seebohm, G., Derst, C., Wei, A.D., Busch, A.E., Steinmeyer, K., 2000. Molecular cloning and functional expression of KCNQ5, a potassium channel subunit that may contribute to neuronal M-current diversity. J. Biol. Chem. 275, 22395–22400.

Marcotti, W., Kros, C.J., 1999. Developmental expression of the potassium current $I_{K,n}$ contributes to maturation of mouse outer hair cells. J. Physiol. 520, 653–660.

Marcotti, W., Johnson, S.L., Holley, M.C., Kros, C.J., 2003. Developmental change in the expression of potassium currents of embryonic, neonatal and mature mouse inner hair cells. J. Physiol. 548.2, 383–400.

Marres, H., van Ewijk, M., Huygen, P., Kunst, H., van Camp, G., Coucke, P., Willems, P., Cremers, C., 1997. Inherited nonsyndromic hearing loss. An audiovestibular study in a large family with autosomal dominant progressive hearing loss related to DFNA2. Arch. Otolaryngol. Head Neck Surg. 123, 573–577.

Nakagawa, T., Kakehata, S., Yamamoto, T., Akaike, N., Komune, S., Uemura, T., 1994. Ionic properties of I_K, n in outer hair cells of guinea pig cochlea. Brain Res. 661, 293–297.

Nouvian, R., Ruel, J., Wang, J., Guitton, M.J., Pujol, R., Puel, J.L., 2003. Degeneration of sensory outer hair cells following pharmacological blockade of cochlear KCNQ channels in the adult guinea pig. Eur. J. Neurosci. 17, 2553–2562.

Oliver, D., Knipper, M., Derst, C., Fakler, B., 2003. Resting potential and submembrane calcium concentration is inner hair cells in the isolated mouse cochlea are set by KCNQ-type potassium channels. J. Neurosci. 23, 2141–2149.

Ryan, A., Dallos, P., 1975. Effect of absence of cochlear outer hair cells on behavioural auditory threshold. Nature 253, 44–46.

Schroeder, B.C., Hechenberger, M., Weinreich, F., Kubisch, C., Jentsch, T.J., 2000. KCNQ5, a novel potassium channel broadly expressed in brain, mediates M-type currents. J. Biol. Chem. 275, 24089–24095.

Talebizadeh, Z., Kelley, P.M., Askew, J.W., Beisel, K.W., Smith, S.D., 1999. Novel mutation in the KCNQ4 gene in a large kindred with dominant progressive hearing loss. Hum. Mutat. 14, 493–501.

Van Camp, G., Coucke, P.J., Akita, J., Fransen, E., Abe, S., De Leenheer, E.M., Huygen, P.L., Cremers, C.W., Usami, S., 2002. A mutational hot spot in the KCNQ4 gene responsible for autosomal dominant hearing impairment. Hum. Mutat. 20, 15–19.

Van Hauwe, P., Coucke, P.J., Ensink, R.J., Huygen, P., Cremers, C.W., Van Camp, G., 2000. Mutations in the KCNQ4 K+ channel gene, responsible for autosomal dominant hearing loss, cluster in the channel pore region. Am. J. Med. Genet. 93, 184–187.
Wang, H.S., Pan, Z., Shi, W., Brown, B.S., Wymore, R.S., Cohen, I.S., Dixon, J.E., McKinnon, D., 1998. KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. Science 282, 1890–1893.
Yang, S.M., Jing, S., Doi, T., Kaneko, T., Yamashita, T., 2002. Isolation of guinea pig inner hair cells using manual microsurgical dissection. J. Oto-Rhino-Laryngol. 64, 1–5.
Zhang, Z., Zeng, X.H., Xia, X.M., Lingle, C.J., 2009. N-terminal inactivation domains of beta subunits are protected from trypsin digestion by binding within the antechamber of BK channels. J. Gen. Physiol. 133, 263–282.

Effect of hydrogen peroxide on potassium currents in inner hair cells isolated from guinea pig cochlea

Takashi Kimitsuki, Noritaka Komune, Teppei Noda, Kazutaka Takaiwa, Mitsuru Ohashi and Shizuo Komune

Hydrogen peroxide (H_2O_2) is a ubiquitous reactive oxygen species that can induce several inner ear disorders. In this study, we recorded the potassium (K) currents in acutely isolated inner hair cells of guinea pig cochlea, and investigated the effects of H_2O_2. We also observed the morphological changes in inner hair cells induced by H_2O_2. In the H_2O_2 solutions, the amplitude of outward K currents ($I_{K,f}$ and $I_{K,s}$) clearly decreased after perfusion for approximately 15 min. Despite the decrease in outward currents, small inward currents ($I_{K,n}$) did not show any reduction. H_2O_2 induced morphological changes in the inner hair cells. All the inner hair cells in the H_2O_2 solutions showed shrinkage and granularity of the cell body and led to loss of viability. These results showed the vulnerability of inner hair cells to reactive oxygen species-induced inner ear disorders. *NeuroReport* 21:1045–1049 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

NeuroReport 2010, 21:1045–1049

Keywords: inner ear, ion current, morphology, organ of corti, reactive oxygen species

Department of Otolaryngology, Graduate School of Medical Sciences, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Correspondence to Dr Takashi Kimitsuki, Department of Otolaryngology, Graduate School of Medical Sciences, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
Tel: +81 92 642 5668; fax: +81 92 642 5685;
e-mail: kimitaka@qent.med.kyushu-u.ac.jp

Received 12 July 2010 accepted 23 August 2010

Introduction

Reactive oxygen species (ROS) have been postulated to be involved in disorders of the inner ear, such as ischemic impairment [1], presbycusis [2], acoustic trauma [3], and labyrinthitis [4]. ROS are also involved in drug-induced hearing impairment. Gentamicin, an aminoglycoside antibiotic, produced ROS, and free radicals were detected in auditory hair cells after gentamicin treatment [5]. Cisplatin damages hair cells by lowering the antioxidant defense system of the inner ear [6]. In humans, the production of superoxide in inner ear perilymph has recently been reported in profound hearing loss [7].

Hydrogen peroxide (H_2O_2) is a ubiquitous ROS that can easily penetrate the cell membranes and is converted to the hydroxyl radical in the presence of Fe^{2+}. In addition to the transition of hydroxyl radical formation, an intracellular Ca^{2+} was elevated [8], which has been suggested to be involved in H_2O_2 toxicity [9]. H_2O_2-induced morphological changes, including bleb formation at the synaptic pole and shortening of the major axis of the cells has been observed in both cochlear hair cells [10,11] and vestibular hair cells [12].

In the mammalian cochlea, inner hair cells receive nearly all the afferent innervations and are primary acoustic transducers. Several ion channels are involved in the inner hair cell function, that is, receiving the mechanical displacement of stereocilia on the apical surface of the cells generates transmitter release onto auditory nerve endings at the basal pole of the cells. The potassium (K) currents are largest and possess the most robust properties compared with other ion currents, such as mechanoelectrical transducer currents and voltage-gated Ca^{2+} currents. Potassium currents, which determine the resting membrane potentials and allow the cells to perform high-frequency transduction by shortening the membrane time constant [13], are crucial for maintaining the cell physiological functions.

In this study, we isolated the inner hair cell from guinea pig cochlea and identified K currents to evaluate the influence of H_2O_2 on membrane ion channels in inner hair cells. The morphological changes in the inner hair cells by H_2O_2 were also investigated.

Materials and methods

Adult albino guinea pigs (200–350 g) with normal Preyer reflex were killed by rapid cervical dislocation, both bullae were removed and the cochlea was exposed. The cochlea, fused to the bulla, was placed in a Ca^{2+}-free external solution (in mM: 142 NaCl, 4 KCl, 3 $MgCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH). The otic capsule was opened, allowing removal of the organ of Corti attached to the modiolus. The organ of Corti was treated with trypsin (0.5 mg/ml, T-4665; Sigma-Aldrich, Missouri, USA) for 12 min, and gentle mechanical trituration was carried out. Trypsin was rinsed from the specimen by perfusing with a standard external solution (in mM: 142 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 NaH_2PO_4, 8 Na_2HPO_4, adjusted to pH 7.4 with NaOH) for at least 10 min before starting any experiment.

0959-4965 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins

DOI: 10.1097/WNR.0b013e32833fcb56

The most important landmarks for identifying inner hair cells are a tight neck and the angle between the cuticular plate and the axis of the cell.

Membrane currents were measured by conventional whole-cell voltage-clamp recordings using an EPC-10 (HEKA, Lambrecht, Germany). Data acquisition was controlled by the software PatchMaster (HEKA). Recording electrodes were pulled with a two-stage vertical puller (PP830, Narishige, Tokyo, Japan) using a borosilicate glass of an outer diameter of 1.5 mm (GC-1.5, Narishige, Tokyo, Japan) filled with an internal solution (in mM: 144 KCl; 2 $MgCl_2$; 1 NaH_2PO_4; 8 Na_2HPO_4; 2 ATP; 3 D-glucose; 0.5 EGTA; adjusted to pH 7.4 with KOH). Pipettes showed a resistance of 4–8 MΩ in the bath and were coated with ski wax (Tour-DIA, DIAWax, Otaru, Japan) to minimize capacitance. The capacitance of the cell was 11.3 ± 3.2 pF [mean ± standard deviation (SD)] and the series resistance was 16.3 ± 5.4 MΩ ($n = 19$). Hydrogen peroxide (H_2O_2, H1009, Merck, Darmstadt, Germany) was applied under pressure (Pressure micro-injector: PMI-200, Dagan, Minneapolis) using pipettes with a tip diameter of 2–4 µm positioned approximately 50 µm from the inner hair cells. These cells were continuously perfused with external saline and all experiments were performed at room temperature (20–25°C).

Isolated inner hair cells were observed under an inverted microscope (TE2000-U, Nikon, Tokyo, Japan) using a CCD video camera (Sony, XC-ST70/ST70CE, Tokyo, Japan) and images were captured into the video frames (Sony DCR HC62).

The experimental design was reviewed and approved (accession number: A21-085-0) by the Animal Care and Use Committee, Kyushu University. All procedures were conducted in accordance with the Guidelines for Animal Care and Use Committee, Kyushu University.

Results

Membrane currents in response to hyperpolarizing and depolarizing voltage steps from a holding potential of −60 mV were recorded from inner hair cells. Typical current records in standard solutions are shown in Fig. 1a. Inner hair cells showed outwardly rectifying K currents ($I_{K,f}$ and $I_{K,s}$) in response to depolarizing voltage pulses, with only a slight inward current ($I_{K,n}$) when hyperpolarized. After 15 min, the K currents did not show noticeable changes in either amplitude or shape (Fig. 1a in right panel). In the solution of 10 mM H_2O_2, the amplitude of K current decreased after perfusion for 15 min (Fig. 1b). Despite the decrease in outward currents, small inward currents did not show any reduction. The activating kinetics in each voltage-dependent outward K current preserved a fast rising rate (lower enlarged scale in Fig. 1b).

Morphological changes in inner hair cells were observed after the application of 10 mM H_2O_2 with the depression of K currents (Fig. 2). H_2O_2 induced shrinkage and granularity of the cell body after approximately 10 min and led to loss of viability. All four inner hair cells were unable to survive in 10 mM H_2O_2 solutions for 20 min.

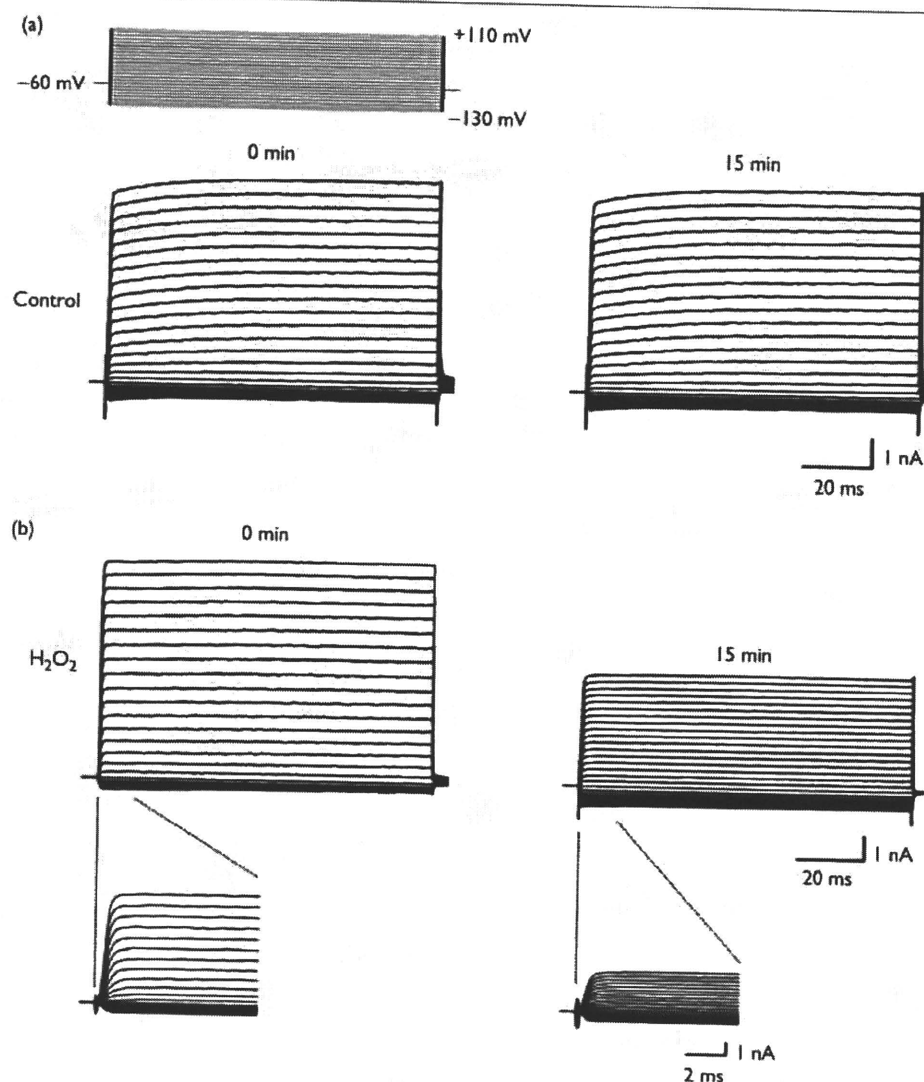
A comparison of the amplitude of outward K currents recorded in control solutions and 10 mM H_2O_2 solutions is shown in Fig. 3. The amplitudes of outward K currents at +110 mV were measured after the application of standard or H_2O_2 solutions for 5, 10, and 15 min and amplitudes relative to those at 0 min were shown from 15 inner hair cells in control and four inner hair cells in H_2O_2 solutions. Five minutes after application, mean ± SDs were 0.97 ± 0.16 and 0.76 ± 0.09 for control and H_2O_2 solutions, respectively, showing a significant difference ($P < 0.05$). After 10 min, mean ± SDs were 0.91 ± 0.14 and 0.66 ± 0.10 for control and H_2O_2 solutions, respectively, showing a significant difference ($P < 0.05$). After 15 min, mean ± SDs were 0.90 ± 0.09 and 0.45 ± 0.12 for control and H_2O_2 solutions, respectively, also showing a significant difference ($P < 0.01$).

Discussion

The effects of H_2O_2 on the K channels in cochlear inner hair cells were studied. H_2O_2 inhibited channel activity and decreased the current amplitudes within 15 min. There was no change in the activating kinetics of the channel (Fig. 1b). H_2O_2 by itself is not sufficiently reactive to oxidize organic molecules in an aqueous environment. Nevertheless, H_2O_2 has the ability to generate highly reactive hydroxyl-free radicals through its interaction with redox-active transitional metals (Fe^{2+} or Cu^{+}, 'Fenton reaction'). Hydroxyl-free radicals oxidize cell membrane lipids and alter cell membrane enzymes and receptors, leading to changes in membrane permeability and the ionic gradient [14]. Although H_2O_2 is a physiologically ubiquitous ROS, an excess production of superoxide in human perilymph of the inner ear with profound hearing loss was established by spectrophotometric analyses [7].

In addition to the 'Fenton reaction' an increase in the cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ has been suggested to be involved in H_2O_2 toxicity [9]. H_2O_2-induced $[Ca^{2+}]_i$ elevation was reported to be in a concentration-dependent manner [8]. Protection by the calcium channel blocker, nifedipine, of the H_2O_2-induced outer hair cell death [11], suggests the participation of voltage-gated Ca^{2+} channels in H_2O_2-induced $[Ca^{2+}]_i$ elevation. An increase of cytosolic calcium has been shown to be involved in the intracellular Ca^{2+} stores [9], which was independent of voltage-gated Ca^{2+} channels [15]. The reduction of K channel activity in this study showed a voltage-independent behavior (Fig. 1b), suggesting the contribution of Ca^{2+} stores. A rise of $[Ca^{2+}]_i$ has been associated with cytotoxic effects, such as destruction of the cytoskeleton, membrane injury, DNA fragmentation,

Fig. 1

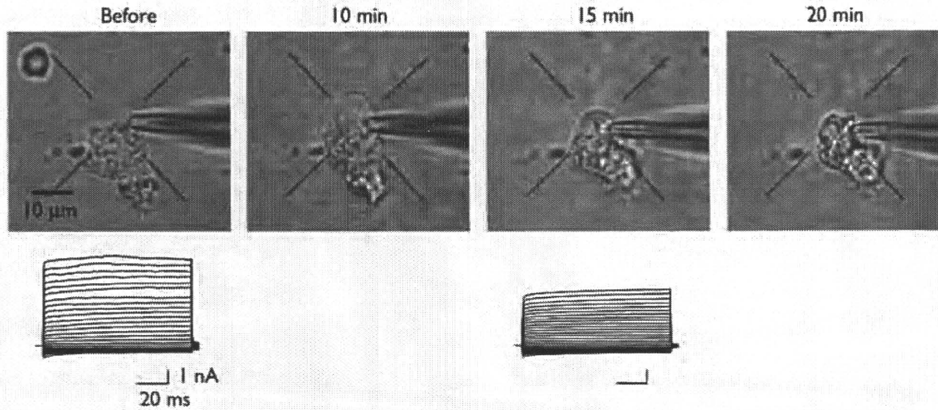


Effects of hydrogen peroxide (H_2O_2) on potassium (K) currents in inner hair cells. (a) K currents in control solutions at 0 and 15 min. The upper panel shows the voltage step protocol. (b) K currents in 10 mM H_2O_2 solutions at 0 and 15 min after application. The lower insets show the initial activating phase in expanded time scales.

and damage to cell organelles such as mitochondria [16]. Exposure of the cells to H_2O_2 results in an increased formation of oxidized protein sulfhydryl groups. The redox state of protein sulfhydryl groups also affects Ca^{2+} homeostasis [17]. Antioxidants, such as glutathione and N-acetylcysteine, suppressed the continuous increase of cytosolic Ca^{2+} and protected against H_2O_2-induced cell damage [18].

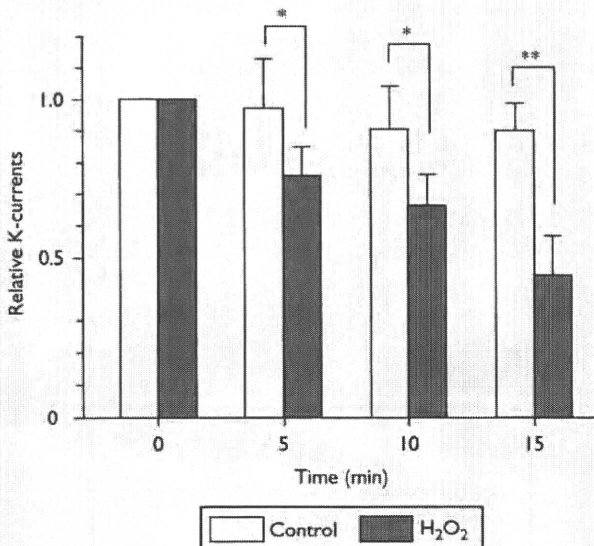
Oxidative stress is related to swelling of the cell body, bleb formation, and shortening of the neck region in vestibular hair cells [12], formation of cytoplasmic blebs at the infranuclear pole, and the diminution of outer hair cells [10]. These morphological changes were concentration-dependent. Although a concentration of 10 mM H_2O_2 is sufficient to produce the morphological changes in the cells, inner hair cells in this study did not show any bleb formations or shortening of the cells, but showed shrinkage and granularity of the cell body. Bleb formation in the cell membrane is considered as the response to altered membrane integrity in the absence of cytoskeletal support elements [10,12]. The cortical lattice is one of the cytoskeletal support elements and has a network of actin filaments with colocation of spectrin [19]. Gamma-actin was particularly associated with the lateral wall of the outer and inner hair cells, but became sparse in areas in which the cortical lattice terminates below the region of the nucleus [20]. The amount and distribution of the cortical lattice are different between the outer and inner hair cells [21], so the alteration of the membrane

Fig. 2



Morphological changes of inner hair cells after the application of 10 mM hydrogen peroxide (H_2O_2). The lower records show the potassium currents before and 15 min after the H_2O_2 application. H_2O_2 induced shrinkage and granularity of the cell body.

Fig. 3



Comparison of the amplitude change in outward potassium (K) currents between control and 10 mM hydrogen peroxide solutions. Test pulse voltage was +110 mV. *$P<0.05$, **$P<0.01$.

integrity by H_2O_2 might also be different, causing differences in the pattern of morphological changes between the outer and inner hair cells.

In the inner hair cells, three types of potassium currents ($I_{k,f}$, $I_{k,s}$, and $I_{k,n}$) were distinguishable [13]. The fast-activating current, $I_{k,f}$, was blocked by tetraethylammonium but was resistant to 4-aminopyridine. The properties of $I_{k,f}$ in the inner hair cells resemble the Ca^{2+}-activated K^+ [big potassium (K), BK] currents because of their kinetics and pharmacology [22], and the K^+ currents in the inner hair cells are potentiated by increasing intracellular Ca^{2+} [23]. Ca^{2+}-activated K^+ channels expressed in *Xenopus* oocytes were inhibited by oxidation with H_2O_2 [24,25]. They reported that H_2O_2 decreased both the open channel probability and the number of active channels based on noise analysis of macroscopic currents. H_2O_2 did not affect channel activity when added to the extracellular side, providing evidence for an intracellular site of H_2O_2 action [24]. H_2O_2 also accelerated the rate of channel 'run-down' [25]. In this study, the channel activity inhibited by H_2O_2 was time-dependent (Fig. 3), which might represent the time course of H_2O_2 uptake into the cell or represent the process of K channel 'run-down'.

Conclusion

In cochlear inner hair cells, H_2O_2, a ubiquitous ROS, inhibited the activation of outward K currents after perfusion of approximately 15 min. H_2O_2 induced acute morphological changes in inner hair cells such as shrinkage and granularity of the cell body, leading to loss of viability. Inner ear disorders involving ROS could be initially elicited by functional and morphological changes in the inner hair cell.

Acknowledgement

This study was supported by a Grant-in-Aid for Scientific Research 21592160 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Maetani T, Hakuba N, Taniguchi M, Hyodo J, Shimizu Y, Gyo K. Free radical scavenger protects against inner hair cell loss after cochlear ischemia. *Neuroreport* 2003; **14**:1881–1884.
2 Staecker H, Zheng QY, Van de Water TR. Oxidative stress in aging in the C57B16/J mouse cochlea. *Acta Otolaryngol* 2001; **121**:666–672.
3 Yamasoba T, Schacht J, Shoji F, Miller JM. Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. *Brain Res* 1999; **815**:317–325.

4 Takumida M, Popa R, Anniko M. Lipopolysaccharide-induced expression of reactive oxygen species and peroxynitrite in the guinea pig vestibular organ. *ORL J Otorhinolaryngol Relat Spec* 1998; **60**:254–262.
5 Hirose K, Hockenbery DM, Rubel EW. Reactive oxygen species in chick hair cells after gentamicin exposure *in vitro*. *Hear Res* 1997; **104**:1–14.
6 Lautermann J, Crann SA, McLaren J, Schacht J. Glutathione-dependent antioxidant systems in the mammalian inner ear: effects of aging, ototoxic drugs and noise. *Hear Res* 1997; **114**:75–82.
7 Ciorba A, Gasparini P, Chicca M, Pinamonti S, Martini A. Reactive oxygen species in human inner ear perilymph. *Acta Otolaryngol* 2010; **130**:240–246.
8 Ishii M, Shimizu S, Hara Y, Hagiwara T, Miyazaki A, Mori Y, *et al.* Intracellular-produced hydroxyl radical mediates H_2O_2-induced Ca^{2+} influx and cell death in rat beta-cell line RIN-5F. *Cell Calcium* 2006; **39**:487–494.
9 Hyslop PA, Hinshaw DB, Schraufstatter IU, Sklar LA, Spragg RG, Cochrane CG. Intracellular calcium homeostasis during hydrogen peroxide injury to cultured P388D1 cells. *J Cell Physiol* 1986; **129**:356–366.
10 Clerici WJ, DiMartino DL, Prasad MR. Direct effects of reactive oxygen species on cochlear outer hair cell shape *in vitro*. *Hear Res* 1995; **84**:30–40.
11 Dehne N, Lautermann J, Ten Cate WJ, Rauen U, De Groot H. *In vitro* effects of hydrogen peroxide on the cochlear neurosensory epithelium of the guinea pig. *Hear Res* 2000; **143**:162–170.
12 Tanigawa T, Tanaka H, Hayashi K, Nakayama M, Iwasaki S, Banno S, *et al.* Effects of hydrogen peroxide on vestibular hair cells in the guinea pig: importance of cell membrane impairment preceding cell death. *Acta Otolaryngol* 2008; **128**:1196–1202.
13 Kros CJ, Crawford AC. Potassium currents in inner hair cells isolated from the guinea-pig cochlea. *J Physiol* 1990; **421**:263–291.
14 Halliwell B. Reactive oxygen species and the central nervous system. *J Neurochem* 1992; **59**:1609–1623.
15 Kimura M, Maeda K, Hayashi S. Cytosolic calcium increase in coronary endothelial cells after H_2O_2 exposure and the inhibitory effect of U78517F. *Br J Pharmacol* 1992; **107**:488–493.
16 Orrenius S, McConkey DJ, Bellomo G, Nicotera P. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol Sci* 1989; **10**:281–285.
17 Bellomo G, Orrenius S. Altered thiol and calcium homeostasis in oxidative hepatocellular injury. *Hepatology* 1985; **5**:876–882.
18 Lomonosova EE, Kirsch M, De Groot H. Calcium vs. iron-mediated processes in hydrogen peroxide toxicity to L929 cells: effects of glucose. *Free Radic Biol Med* 1998; **25**:493–503.
19 Slepecky NB, Ulfendahl M. Actin-binding and microtubule-associated proteins in the organ of Corti. *Hear Res* 1992; **57**:201–215.
20 Furness DN, Katori Y, Mahendrasingam S, Hackney CM. Differential distribution of beta- and gamma-actin in guinea-pig cochlear sensory and supporting cells. *Hear Res* 2005; **207**:22–34.
21 Furness DN, Hackney CM. Comparative ultrastructure of subsurface cisternae in inner and outer hair cells of the guinea pig cochlea. *Eur Arch Otorhinolaryngol* 1990; **247**:12–15.
22 Art JJ, Fettiplace R. Variation of membrane properties in hair cells isolated from the turtle cochlea. *J Physiol* 1987; **385**:207–242.
23 Dulon D, Sugasawa M, Blanchet C, Erostegui C. Direct measurement of Ca^{2+}-activated K^+ currents in inner hair cells of the guinea-pig cochlea using photolable Ca^{2+} chelators. *Pflüg Arch* 1995; **430**:365–373.
24 Soto MA, González C, Lissi E, Vergara C, Latorre R. Ca^{2+}-activated K^+ channel inhibition by reactive oxygen species. *Am J Physiol Cell Physiol* 2002; **282**:C461–C471.
25 DiChiara TJ, Reinhart PH. Redox modulation of hslo Ca^{2+}-activated K^+ channels. *J Neurosci* 1997; **17**:4942–4955.