Function and expression of epithelial sodium channel (ENaC) α,β,γ in human endolymphatic sac

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Function and expression of epithelial sodium channel (ENaC) α,β,γ in human endolymphatic sac

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This certifies that the Master's Thesis

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Finally, I hope that I will do an in-depth study for the next years without indolence and conceit, and that every person around me will encourage me doing so.

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ABSTRACT

Function and expression of epithelial sodium channel (ENaC) α,β,γ in human endolymphatic sac

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Ion channels are thought to be essential for maintaining an inner ear homeostasis and regulating volume of endolymph. As water accompanies the Na^+ transport usually, endolymph volume regulation may be related to Na^+ transport in the inner ear, especially in the endolymphatic sac, although of which the exact funtion has not been defined, known to be associated with endolymph volume regulation. There have been many studies about Na^+ transport which might be essential for the homeostasis and volume regulation of the cochlea and vestibular system, but involvement of the endolymphatic sac epithelium in ion transport has recently been studied in animal models. Epithelial Na^+ channel (ENaC), Na^+-H^+-exchanger (NHE), Na^+-K^+-ATPase have been shown to be present in the epithelial cells of the endolymphatic sac by animal models, and Na^+ transport may be accomplished through ENaC and NHE with excretion via Na^+-K^+-ATPase. In the present study, we demonstrated the presence of ENaC in the endolymphatic sac cells by in vitro and in vivo study of RT-PCR and

immunohistochemistry. And we also demonstrated ENaC activities in cultured endolymphatic sac cells by measuring the short circuit current (I_{sc}) in Ussing chamber which decreased by luminal addition of ENaC inhibitor, amiloride (100 μM). This study suggests that ENaC in the apical membrane of the epithelial cells of human endolymphatic sac may plays an important role in regulating inner ear fluid volume and inner ear homeostasis, and also may help elucidating the mechanism of endolymphatic hydrops and Meniere's disease

Key Words : endolymphatic sac, fluid transport, sodium, epithelial sodium channel

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I. INTRODUCTION

Cochlear and vestibular endolymph has an unique composition with a high K^+ concentration (about 160 mM and 150 mM in each compartment) and a low Na^+ concentration (about 1.5 mM and 9 mM) [1], but in the endolymphatic sac, the endolymph has high Na^+ (100 ~ 130 mM) and low K^+ (12~17 mM) concentration [2,3]. These uncommon ion composition and homeostasis of endolymph is believed to be essential for the function of sensory hair cells inner ear, generating endocochlear and endolymphatic potential and keeping inner ear volume constantly.

Transport systems involved in the homeostasis of K^+ concentration in endolymph have been known as belows; K^+ is secreted in endolymph by the marginal cells of the stria vascularis through an atypical K^+ channel coupled with basolateral Na^+-K^+-ATPase and Na^+-K^+-$2Cl^-$ cotransport. K^+ flows from the endolymph through apical transduction channels that are opened by movement of sensory hair cells and recycled toward the stria vascularis through spiral ligament fibroblast via gap junctions [4,5].

Na^+ flux has been poorly understood compared with K^+ transport, but it has been suggested that Na^+ is reabsorbed through epithelial Na^+ channel (ENaC) and Na^+-H^+ exchanger (NHE) located at the apical membrane of the marginal cells of stria vascularis coupled with basolateral Na^+-K^+-ATPase [6,7].

As mentioned above, there are many studies about K^+ and Na^+ transport which are essential for the homeostasis of the cochlea and vestibular system, but involvement of the endolymphatic sac epithelium in ion transport has recently been studied in animal models [8,9]. In case of Na^+ transport, the ENaC and NHE have been shown to be present in the apical membrane of the epithelial cells of the endolymphatic sac [10,11]. Also, the presence of basolateral Na^+-K^+-ATPase has been demonstrated [12,13] and Na^+ transport may be accomplished through ENaC and NHE with excretion via Na^+-K^+-ATPase. As water accompanies Na^+ transport usually, endolymphatic volume regulation may be related to Na^+ transport in the inner ear, especially in the endolymphatic sac, which has been known to be associated with endolymphatic volume regulation in some stuedies[14,15].

The purpose of the present study is to identify Na^+ transport via ENaC in the human endolymphatic sac, which may be contribute to identify the one of causes of endolymphatic hydrops and inner ear homeostatic disorder. Each subunit of α−, β−, γ−ENaC in the human endolymphatic sac were detected by RT-PCR and localized by immunohistochemistry. The function of ENaC was also investigated by measuring short-circuit current, which was inhibited by amiloride, ENaC inhibitor.

II. MATERIALS AND METHODS

1. Cell culture

Endolymphatic sacs of normal appearance were harvested during otologic surgery for acoustic tumor. Tissues were treated with 1% pronase (type XIV protease; Sigma, St. Louis, MO, USA) in a 1:1 mixture of Dulbaco's modified Eagle's medium (DMEM; Gibco. New York, USA) and Ham's nutrient F12 (F12; Gibco) supplemented with penicillin G sodium (50 IU/mL) and streptomycin sulfate (50 μg/ml) for 16 to 20 hours at 4℃. Dissociated epithelial cells were washed 3 times in DMEM/F12 containing antibiotics and suspended in DMEM/F12 supplemented with antibiotics and 10% fetal bovine serum. To remove fibroblast and endothelial cells, isolated cells were placed in a plastic dish and cultured for 30 min at 37 ℃. Suspended epithelial cells were seeded at 3×10^4 cells per dish (500 cells per square centimeter) in 10 cm plastic tissue culture dishes. The culture medium used was a 1:1 mixture of bronchial epithelial growth media (BEGM; Clonetics, Walkersville, MD, USA) and DMEM (Gibco), containing hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 ng/ml), gentamycin (50 μg/ml) and amphotericin B (50 ng/ml), all supplied by Clonetics, and further supplemented with EGF (25 ng/ml; Collaborative Research, Bedford, MA, USA), all-trans retinoic acid (5×10^{-8} M; Sigma, St. Louis, MO, USA), bovine serum albumin (1.5 μg/ml; Sigma), and bovine pituitary extract (1% v/v; Pel Freez, Rogers, AR, USA). The culture medium was changed every other day after the explants established attachment and the outgrowth of cells began. The explant was removed after complete outgrowth of cells and cells were dissociated and harvested with 0.25% trypsin/EDTA (Clonetics).

The cell number was counted with a hemocytometer, and >2000 cells were seeded per square centimeter for subsequent passages. Cells were dissociated again for subcultures when they reached 80% confluency.

2. Measurement of the short circuit current

Cultured cells were mounted in modified Ussing chambers (World Precision Instruments, Sarasota, FL, USA). The epithelium was bathed on both sides with 5 ml of warmed (37°C) regular bicarbonate solution circulated by gas lifts with 95% O_2-5% CO_2. Solution pH was maintained at 7.4. The epithelial culture was voltage clamped with an automatic voltage clamp and the short-circuit current (I_{sc}) was measured. Data were acquired and analyzed with Acquire and Analysis (version 1.2) software. After a 15-min equilibration, amiloride (100 μM) was added to the luminal bath.

3. RT-PCR

Total RNA was prepared from human endolymphatic sac tissue which harvested during acoustic tumor surgery and cultured endolymphatic sac cells using TRIsol reagent according to user's manual, and the concentration determined spectrophotometrically. Total RNA (2 μg) was converted into cDNA using 400 U Moloney murine leukemia virus RT (200 U/μl; Gibco) reverse transcriptase in a buffer containing (in mM) 250 Tris HCL, pH 8.3 275 KCl, 15 MgCl2, and 1 D-nucleotide triphosphate (final volume: 40 μl). The PCRs were performed with a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer, Branchburg, NJ, USA) according to manufacturer's recommendations. The main characteristics of the primers used to amplify the cDNA of the α-, β-, and γ-ENaC subunits are specified in Table. 1. The

cDNA segments were amplified from 1 μl of the RT product in a PCR buffer (in mM: 20 Tris HCl, 50 KCl, 1.5 MgCl2) and in the presence of 0.2 mM of dNTP and 1 U of Taq polymerase at 5 U/μl (Gibco). Each PCR cycle comprised denaturation at 94 ℃ for 30 s, annealing during 30 s at variable temperature (Table 1), and elongation at 72 ℃ for 1 min. 37 cycles were used. The reaction was stopped by a temperature decrease to 4 ℃. A 10-μl sample of the PCR product was run on a 1 % agarose gel with ethidium bromide. A 100-bp molecular weiht ladder permitted the verification of the length of the amplified fragments.

Table 1. Pririmers used for PCR of α-, β-, and ɣ-ENaC subunits

PCR product/Primer Sequence	Segment Amplifed, bp	Anealing Temperature,℃
α-ENaC S: 5'-CAGCCCATACCAGGTCTCAT-3' AS: 5'-ATGGTGGTGTTGTTGCAGAA-3'	220	55
β-ENaC S: 5'-GGGGTACTCGTGGATAAGCTT-3' AS: 5'-GAGACAAGACGTGGAAAATCC-3'	376	57
ɣ-ENaC S: 5'-ACCACCAGCCATGGTCTAAG-3' AS:5'-CTTCAGGTCCCGGGATTTAT-3'	209	54

4. Tissue preparation & immunohistochemistry

Endolymphatic sacs which were harvested during acoustic tumor surgery were fixed in 4% paraformaldehyde solution for 5 hours at 4℃ and the specimens were embedded in paraffin using routine procedures. Some tissues were frozen and evenly coated with OCT compound (Miles, Elkhart, IN, USA). The embedded tissues were sectioned with a rotatory microtome at 5 μm in thickness and mounted on poly-L-lysine-treated glass slides. The paraffin embedded samples were deparaffinized for 5 min in xylene 4 times and rehydrated in decreasing alcohol concentrations. After washing three times in PBS, endogenous peroxidase was blocked by 1 % H_2O_2 in absolute methanol for 20 min. Slides were immersed in 0.01 M sodium citrate buffer (pH6.0) and placed into a microwave oven for 10 min at 92-98℃ to retrieve antigen. Antigen retrieval procedure was omitted in frozen sections. The sections washed three times in PBS and equilibrated with 3% chicken serum (Vector, Burlingame, CA, USA) for 30 min at room temperature to block the nonspecific binding sites. Sections washed three times again and incubated overnight at 4℃ with the primary antibodies which were diluted 1:200, respectively. The ENaC antibodies were provided from Dr. Barbry , Institute de pharmacologie Moleculaire et Cellulaire 660 route des lucioles, France. The antibodies were polyclonal rabbit antibodies against peptide specific to the α, β, γ subunits of ENaC, which have been previously characterized [16]. Following the primary incubation, sections were washed three times in PBS, and then incubated for 1 hour at room temperature with biotin-labeled goat anti-rabbit IgG (Jackson immunoresearch, West grove, PA, USA). Tissues were incubated with streptavidin horseradish peroxidase for 30 min. After washing three times in PBS again, and sites of bound primary antibodies were visualized by monitoring their development in diaminobenzidine substrate

medium (Vector). Sections were examined under Olympus microscope.

In the negative control, sections were processed along the same protocol except omitting the primary antibody in the immunolabeling procedure.

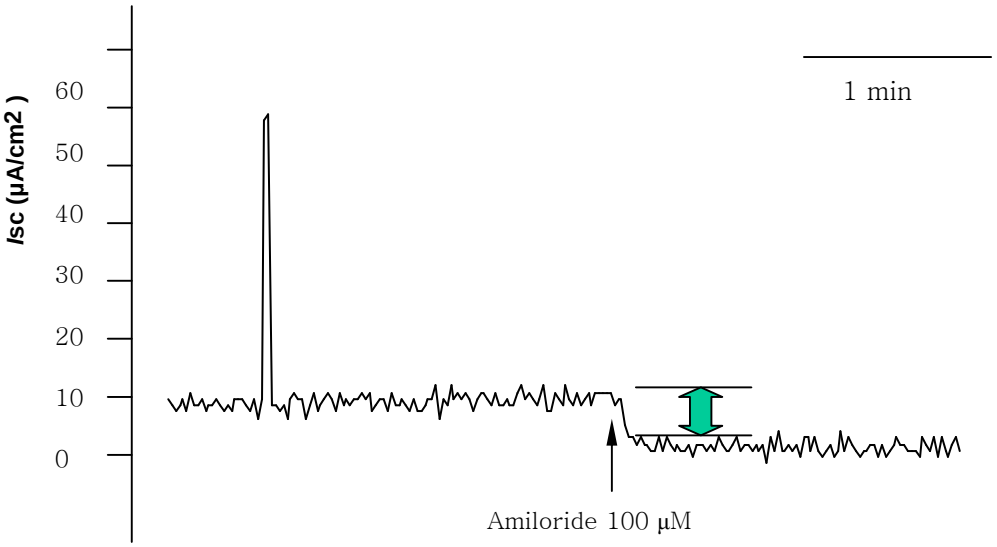
III. RESULTS

1. ENaC- dependent short circuit current

To demonstrate ENaC activities in cultured endolymphatic sac cells, the short circuit current (I_{sc}) was measured. Fig. 1 shows typical I_{sc} traces for determination of ENaC in cultured endolymphatic sac cells. The luminal addition of amiloride (100 μM) produced a rapid decrease of current due to the blockage of ENaC. The cultured cells showed an I_{sc} that was largely inhibited by 100 μM of amiloride**.** The amplitude of the amiloride-sensitive current, which reflects ENaC activity, was 10 $\mu A/cm^2$ in cultured endolymphatic sac cells.

Fig. 1. Amiloride- sensitive current in cultured human endolymphatic sac cells.

Typical short-circuit current (I_{sc}) recording of cultured human endolymphatic sac cells following sequential treatment with 100 μM luminal amiloride

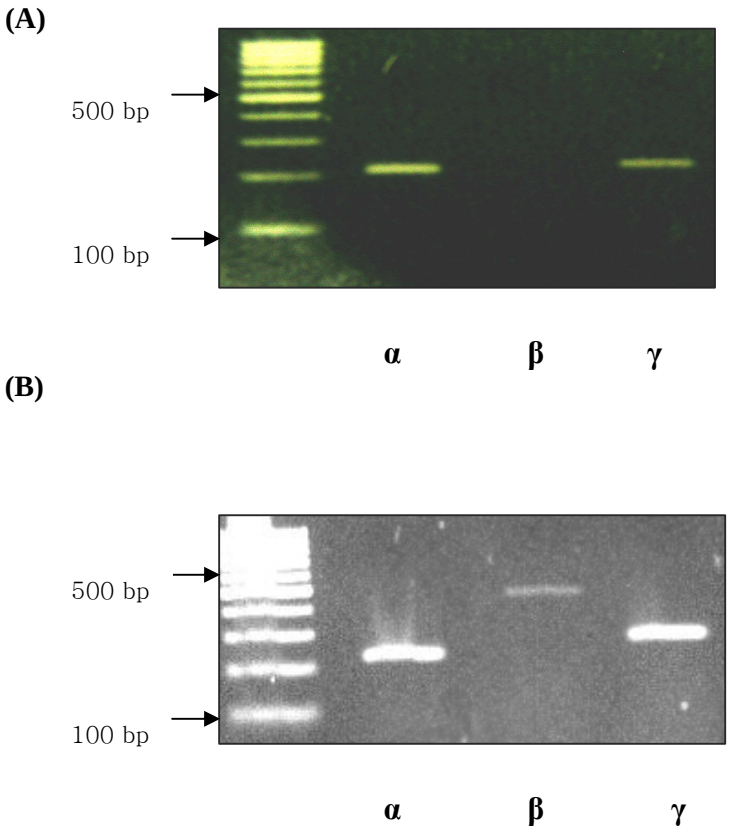


2. Expression of ENaC genes

The RT-PCR product of cultured endolymphatic sac cells yielded bands compatible with the expected sizes of α, β, γ subunits of ENaC and control genes (Fig.2A). In case of human endolymphatic sac tissues, mRNA encoding α and γ subunits of ENaC were detected, but β subunit was not detected (Fig.2B).

Fig. 2. Expression of ENaC genes

The RT-PCR product of human endolymphatic sac tissue (A) and cultured endolymphatic sac cells (B). In case of human endolymphatic sacs tissue, mRNA encoding α and γ subunits of ENaC were detected, but β subunit was not detected. (α : ENaC α, β : ENaC β, γ: ENaC γ)



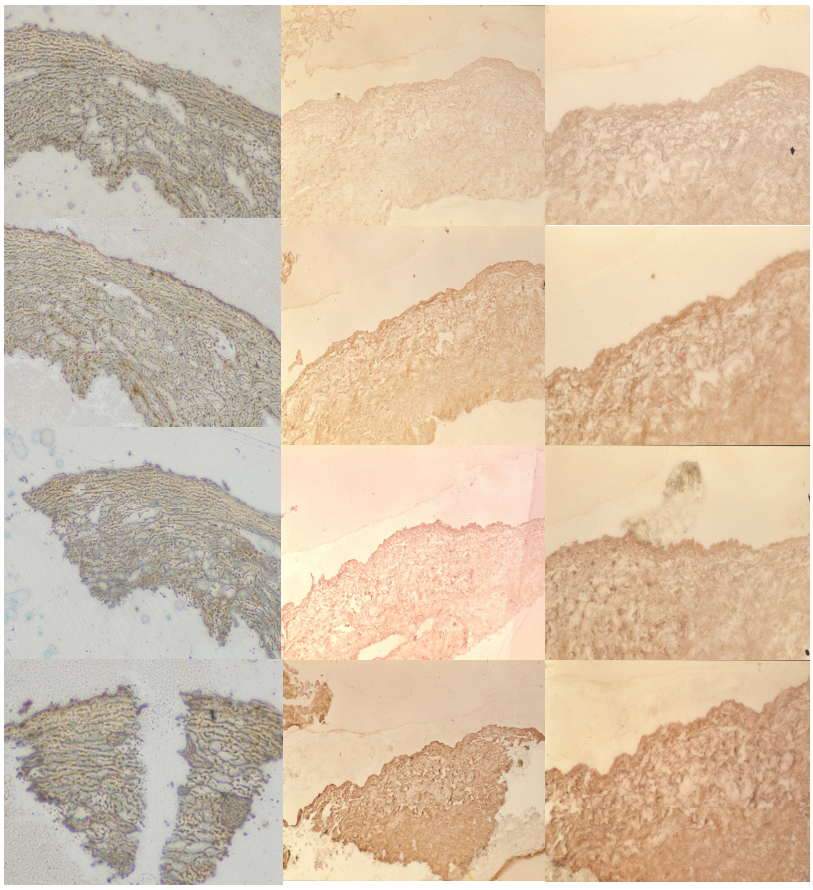
3. Immunohistochemistry

All subunits of ENaC were distributed in the epithelium and the stroma of endolymphatic sac, compared with the control but β subunit was weakly expressed

(Fig.3). The labeling of epithelium was evenly expressed and stronger than that of the stroma of endolymphatic sac

Fig. 3. Immunohistochemistry.

All subunits of ENaC were distributed in the epithelium and stroma of endolymphatic sac, compared with the control, but β subunit was weakly expressed. (* : frozen sectioned)



IV. DISCUSSION

ENaC generally consists of three homologous subunits which assemble into $\alpha_2\,\beta\gamma$ heterotetramer or $\alpha_3\beta_3\gamma_3$ [17,18]. The δ ENaC subunit identified in some organs such as testis, ovary, pancreas, brain and heart, but their physiological role in these tissues remains unknown[19]. ENaC is expressed in a wide variety of epithelia including kidney, sweat glands, lung and gastrointestinal tract and is located in the apical membrane of polarized epithelial cells where it mediates Na^+ transport across tight epithelia. The basic function of ENaC is to allow vectorial transcellular transport of Na^+ by electrochemical gradient coupled with the Na^+-K^+-ATPase of basolateral membrane and the apical entry of Na^+ is blocked by submicromolar concentrations of amiloride [6]. This active transepithelial transport of Na^+ is important for maintaining the composition and the regulating volume of the fluid on either side of the epithelium.

At least five morphologically different types of cells consist of human endolymphatic sac epithelium. They are flat, cuboidal, columnar and have dark or clear cytoplasm. The functions of cells were not demonstrated, but they were thought to have functions of phagocytosis, ion and water transport and secretion [20].

Endolymphatic sac has been known to regulate inner ear volume [14,15] and the epithelial cells of the sac might have an important role in the regulation. Disturbances in the function of the endolymphatic sac are believed to be involved in the genesis of inner ear disorders such as endolymphatic hydrops and Meniere's disease [14,15], although it might not be the sole cause of disease. However, the pathophysiology of the development of endolymphatic hydrops is still unclear.

The involvement of the endolymphatic sac epithelium in ion and water transport was

recently been studied. The presence of ENaC, NHE, Na^+-K^+-ATPase and aquaporins in the epithelium of endolymphatic sac were demonstrated in some studies with animal models [10,11,12,13,21]. This is the first study which demonstrate the presence of ENaC in the human endolymphatic sac. In the present study, ENaC-dependent I_{sc} was prominent and ENaC subunit gene expressions were detected in the cultured cells. But in the RT-PCR of human endolymphatic sac tissues, β subunit of ENaC was nearly not expressed compared with that of cultured cells. Also, in immunohistochemistry α, γ subunit of ENaC were expressed in the epithelium, but β subunit was expressed very weakly. These results suggest that ENaC which located at human endolymphatic sac might have some structural difference from that of other organs. It also might be resulted from different cell types between in vitro and in vivo epithelial cells. The ultrastructural study will be needed to demonstrate the structure and characteristics of ENaC in human endolymphatic sac. And more functional study of ENaC in each different epithelial cell types will be needed to elucidate the part of the ion and water transport mechanism in human endolymphatic sac.

The immunohistochemistry showed subunits of ENaC distributed evenly in the epithelial cells and the stroma also showed weak expression of immunolabeling of ENaC. It seems that Na^+ transport might be mediated mainly by ENaC which presents in the apical membrane of epithelial cells, but ENaC is might present in the fibroblast or connective tissues of the endolymphatic sac. The presence of ENaC in nonepithelial tissue was demonstrated in some studies[22,23,24], but their role was not clearly defined. Futher functional studies of nonepithelial tissue, such as fibroblast will be needed to clear the role of ENaC in nonepithelial tissues.

V. CONCLUSION

We have demonstrated an enhanced amiloride-dependent $\boldsymbol{I_{sc}}$ and presence of each subunits of ENaC in the human endolymphatc sac *in vivo and in vitro*. Although Na^+ transport might be mediated by other mechanisms such as NHE, Na^+-K^+-ATPase and Na^+-K^+-Cl^- cotransporter, ENaC may play an important role in Na^+ transport between epithelial cells and endolymph. It means that ENaC may be involved in the inner ear fluid regulation and homeostasis, thus the result of the present study may help elucidating the mechanism of endolymphatic hydrops and Meniere's disease

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국문요약

사람 내림프낭에서의 epithelial sodium channel (ENaC) α,β,γ의 기능과 발현

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의학과

(지도교수 : 이 원 상)

이온 채널은 내이의 항상성과 내림프의 양을 조절하는 데에 있어 필수적인 것으로 사료되어진다. 일반적으로 Na^+의 이동은 물의 이동을 수반하므로, 내림프의 양의 조절은 내이에서의 Na^+ 이동과 밀접한 관계가 있다고 할 수 있다. 특히 내림프낭은 현재까지 그 명확한 기능이 밝혀지지는 않았지만, 내림프 양의 조절과 관련이 있다고 알려져 있으며, 이러한 내림프낭에서의 Na^+이동 또한 내림프 양의 조절과 밀접한 연관이 있을 것으로 사료된다. 와우와 전정의 내림프에서 Na^+이동에 대해서는 많은 연구가 이루어졌지만, 내림프낭상피에서의 이온 수송에 대해서는 근래에 와서야 동물실험을 통하여 연구가 되어지기 시작하였다. Epithelial Na^+ channel (ENaC), Na^+-H^+-exchanger, Na^+-K^+-ATPase가 내림프낭의 상피에 존재함이 동물 실험으로 증명되었으며, Na^+의 이동은 타 부위와 마찬가지로 상피세포 첨부의 ENaC과 NHE로 흡수되어 기저면의 Na^+-K^+-ATPase로 배출되는 것으로 사료된다. 본 연구에서는 정상 내림프낭 조직과 배양된 내림프낭 세포에서 RT-PCR과 면역조직화학염색을 이용하여 내림프낭상피에서의 ENaC의 존재를 규명하고, 배양된 상피에서 Ussing chamber를 이용하여 short-circuit

current(I_{SC})의 발생과 ENaC 길항제인 amiloride (100 μM)가 이를 차단함을 보여 ENaC의 기능을 입증하였다. 이러한 결과는 사람 내림프낭상피의 첨부에 존재하는 ENaC이 내림프의 양 조절과 항상성에 중요한 역할을 담당함을 시사하며, 내림프수종과 메니에르병의 병태생리 규명에 기여할 수 있을 것으로 사료된다.

핵심되는 말 : 내림프낭, 내림프 유입, sodium, epithelial sodium channel