Location and function of the epithelial Na channel in the cochlea

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1Institut National de la Santé et de la Recherche Médicale U-426 and 2U-478, Faculté Xavier Bichat, 75870 Paris Cedex 18; and 3Department of Oto-Rhino-Laryngology, Hôpital Beaujon, AP-HP, 92118 Clichy, France

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Couloigner, Vincent, Michel Fay, Sabri Djelidi, Nicolette Farman, Brigitte Escoubet, Isabelle Runembert, Olivier Sterkers, Gérard Friedlander, and Evelyne Ferrary. Location and function of the epithelial Na channel in the cochlea. Am J Physiol Renal Physiol 280: F214–F222, 2001.—In the cochlea, endolymph is a K-rich and Na-poor fluid. The purpose of the present study was to check the presence and to assess the role of epithelial Na channel (ENaC) in this organ. α-, β-, and γ-ENaC subunit mRNA, and proteins were detected in rat cochlea by RT-PCR and Western blot. α-ENaC subunit mRNA was localized in situ hybridization in both epithelial (stria vascularis, spiral prominence, spiral limbus) and nonepithelial structures (spiral ligament, spiral ganglion). The α-ENaC-positive tissues were also positive for β-subunit mRNA (except spiral ganglion) or for γ-subunit mRNA (spiral limbus, spiral ligament, and spiral ganglion), but the signals of β- and γ-subunits were weaker than those observed for α-subunit. In vivo, the endo cochlear potential was recorded in guinea pigs under normoxic and hypoxic conditions after endolymphatic perfusion of ENaC inhibitors (amiloride, benzamil) dissolved either in K-rich or Na-rich solutions. ENaC inhibitors altered the endocochlear potential when Na-rich but not when K-rich solutions were perfused. In conclusion, ENaC subunits are expressed in epithelial and nonepithelial cochlear structures. One of its functions is probably to maintain the low concentration of Na in endolymph. 

endolymph; stria vascularis; in situ hybridization; endocochlear potential; hypoxia

IN THE COCHLEA, THE MEMBRANOUS labyrinth is a complex sensorineural epithelium composed of sensory hair cells and their supporting cells and of various nonsensory epithelia, namely, the stria vascularis lined by the connective tissue of the spiral ligament, the spiral prominence, the outer sulcus cells, the spiral limbus, and Reissner’s membrane (Fig. 1). The basolateral side of the membranous labyrinth is bathed by perilymph, an extracellular fluid whose chemical composition is similar to that of blood and cerebrospinal fluid (35). Apically, the fluid that bathes the hair bundles of sensory cells is called endolymph and has a unique composition among mammalian extracellular fluids. It is K rich (170 mM), almost devoid of Na (1 mM), and hyperosmolar (30–40 mM over blood osmolality) (35). The transepithelial potential between perilymphatic and endolymphatic compartments is +100 mV, endolymph positive. Homeostasis of the volume, pressure, and electrochemical composition of endolymph is necessary to the mechanoelectrical transduction, i.e., the transformation of sound into nerve impulse through the apical tranduction channels of the sensory hair cells. Transport systems involved in the homeostasis of K concentration in endolymph have been mainly elucidated. K is secreted in endolymph by the marginal cells of the stria vascularis through an apical IsK/KvLQT1 K channel coupled with basolateral Na+,K+-ATPase and Na-K-Cl cotransport (39). It is transported out of endolymph by hair cell apical tranduction channels and probably by outer sulcus cells’ apical nonselective cationic channels (23), and is then probably recycled toward the stria vascularis through spiral ligament fibroblasts (34, 37).

At variance with K flux, the pathway of cochlear transepithelial Na flux remains poorly understood. The Na electrochemical gradient that takes place between endolymph and perilymph suggests that the membranous labyrinth is an Na-reabsorbing epithelium. Koefoed-Johnsen and Ussing (19) showed that Na is reabsorbed through high-resistance epithelia by functional coupling between passive Na diffusion through the apical membrane and active extrusion of intracellular Na by basolateral Na+,K+-ATPase. In the cochlea, basolateral Na+,K+-ATPase has been detected in several epithelial cells such as stria vascularis marginal cells (35) and outer sulcus cells (27). The apical transport system responsible for Na reabsorption from endolymph remains unknown. A good candidate would be the epithelial Na channel, ENaC. Indeed, this channel has been shown to participate to transepithelial Na transport in many epithelia such
as kidney, colon, lung, sweat glands, and salivary glands (2, 13, 15).

The purpose of the present study was to detect the presence of α-, β-, and γ-ENaC subunit mRNA and proteins in the rat cochlea by RT-PCR and Western blot and to localize ENaC mRNA within the rat cochlea by in situ hybridization. The function of cochlear ENaC was investigated in vivo in guinea pigs by assessing the effect of locally applied ENaC inhibitors, amiloride and benzamil, on the endocochlear potential.

METHODS

The procedures concerning animals reported in the present work were approved by Ministère de l’Agriculture et de la Forêt (approval no. 5521).

RT-PCR

RNA extraction from rat cochlea. Two Long-Evans rats (Janvier, Le Genest, France) were killed with a lethal injection of pentobarbital sodium (100 mg/kg ip) and quickly decapitated. The four temporal bones were rapidly removed, and the cochlear soft tissues were microdissected in a 4°C PBS (Dulbecco’s, without calcium, magnesium, and sodium bicarbonate, (GIBCO-BRL, Life Technologies, Gaithersburg, MD)). Microdissected tissues were pooled and transferred in 100 μl of lysis buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-laurylsarcosine, 0.1 M β-mercaptoethanol) and mechanically minced. Total mRNA was extracted with phenol water and isoamyl alcohol-chloroform (1:24, vol/vol) and precipitated by isopropanol (8). The pellet was resuspended in 20 μl of water. Genomic DNA was eliminated by adding 4 μl of 5× transcription buffer (GIBCO-BRL), 0.5 μl (10 U/μl) of RNase-free DNase I (Amersham Pharmacia Biotech, Piscataway, NJ), and 1 μl of RNasin (40 U/μl; Promega, Madison, WI). This solution was heated for 60 min at 37°C. An ethanol precipitation was then performed, and the pellet was dried and resuspended in 20 μl of water.

Reverse transcription of RNA extracted from rat cochlea. cDNA was synthetized by reverse transcription of RNA in solution (2 μl RNA solution extracted from rat cochlea) with 400 U Moloney murine leukemia virus RT (200 U/μl; GIBCO-BRL) 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). To verify the possibility of genomic DNA amplification during the PCR reaction, RT negative controls were performed by omitting the RT in the solution.

PCR. The main characteristics of the primers used to amplify the cDNA of the α-, β-, and γ-ENaC subunits are specified in Table 1. β- and γ-ENaC primers, but not α-primers, amplified cDNA fragments whose corresponding genomic DNA encompassed at least one intron. The cDNA segments were amplified from 10 μl of the RT product or of the RT-negative control 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

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Fig. 1. Cross section of the scala media. Cochlear fluids encompass the perilymph in the scalae vestibuli and tympani, a Na-rich and K-poor fluid, and the endolymph in the scala media, a K-rich and Na-poor fluid. The scala media is lined by several epithelial structures. Inferiorly, the fibrous basilar membrane (BM) supports the organ of Corti (OC), the sensory organ of the cochlea, and the spiral limbus, involved in the maintenance of endolymphatic pH. Superiorly, Reissner’s membrane (RM) is composed of 2 cell layers. Laterally, the stria vascularis (SV), responsible for endolymphatic K secretion, and the spiral prominence (SP), whose function remains to be determined, both lie on a connective tissue called the spiral ligament (SL). The spiral ligament is thought to participate in the recycling of K from the basolateral side of sensory hair cells to the stria vascularis. The tectorial membrane (TM) is in contact with the apical hair bundles of the sensory cells and thus participates in the mechanoelectrical transduction process. The somatic body of the neurons that innervate the sensory hair cells are located in the spiral ganglion.
1 U of Taq polymerase at 5 U/µl (GIBCO-BRL). Each PCR cycle comprised denaturation at 94°C for 30 s, annealing during 30 s at variable temperatures (Table 1), and elongation at 72°C for 1 min. Forty cycles were used. The reaction was stopped by a temperature decrease to 4°C. A 10-µl sample of the PCR product was run on a 1% agarose gel with ethidium bromide. A 100-bp molecular weight (MW) ladder permitted the verification of the length of the amplified fragment. The whole procedure was repeated in three series of two animals.

In Situ Hybridization

The temporal bones of Long-Evans rats were rapidly removed (see above) and immersed in a solution of 4% paraformaldehyde (PFA) in PBS (0.1 M, pH 7.4). The cochleas were dissected in PFA, and the round and oval windows were opened to facilitate PFA entry in the cochlea. Cochleas were fixed in PFA for 24 h, decalcified during 21 days in 8% EDTA, and paraaffin embedded. In situ hybridization was performed on 7-µm sections (Leitz) with radiolabeled riboprobes. To prepare riboprobes, part of the 3'-untranslated region to the α-, β-, and γ-subsites of rat ENaC (corresponding to nucleotides 2185–2775 for the α-subunit, 2150–2463 for the β-subunit, and 2470–2911 for the γ-subunit) (10) were subcloned into pBluescript KS+ vector and linearized with BamHI or KpnI. Probes were synthesized by using a T3/T7 in vitro RNA synthesis kit (Promega) in the presence of 35S-labeled UTP (Amersham). Hybridization was essentially done as previously described (10). In brief, paraaffin sections were postfixed in 4% PFA, treated with proteinase K, acetylated, covered with the hybridization mixture containing the 35S-labeled probe, and incubated overnight at 50°C. Washing was done with 5× saline sodium citrate (SSC) and 10 mM dithiothreitol (DTT) at 50°C for 30 min, followed by a high-stringency wash in 50% formamide, 2× SSC, and 0.1 M DTT at 65°C for 20 min, then several washes in NaCl-Tris-EDTA (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) at 37°C. After subsequent RNase treatment, sections were rinsed in 0.1× SSC for 15 min, dehydrated, and dried. For autoradiographic detection, slides were dipped in Kodak NTB2 photoemulsion (Kodak, Rochester, NY), dried, and exposed at 4°C for 6 wk. Before examination, slides were counterstained with 1% toluidine blue. Control experiments were done by using sense probes for the respective cRNA probes. The signal intensity was semiquantitatively assessed by using the Scion image analysis software (Scion, Frederick, MD). Image analysis was performed for each subunit on two cochleas originating from two different rats. For each probe, the signal intensity was corrected by subtracting the background signal intensity determined in the inner ear fluid compartments (scala vestibuli, scala tympani, scala media; see Fig. 1). To compare the antisense-specific signal and the sense nonspecific signal, the ratio (antisense probe signal intensity−sense probe signal intensity)/sense probe signal intensity) was calculated. ENaC subunit mRNA was considered to be present when this ratio was superior to 25%.

Western Blot

Western blot was performed on 14 cochlear soft tissues microdissected from 7 Long-Evans rats. Cochlear proteins homogenates were extracted from a 200-µl solution containing (in mM) 50 Tris-HCl, pH 7.5, 150 NaCl, 1% Triton X-100, and 5 EDTA.

SDS-PAGE was performed with solubilized membranes (15–63 μg of proteins) and prestained MW markers (Sigmal, 3% polyacrylamide minigels (Mini PROTEAN II, Bio-Rad, Hercules, CA). Proteins were subsequently transferred from the gels electrophoretically to nitrocellulose membranes (Mini Trans-Blot Module, Bio-Rad). Transfer efficiency was checked by Ponceau red staining of the nitrocellulose membranes.

To detect α-, β-, and γ-ENaC subunits, rabbit monoclonal antibodies, whose specificity had been previously tested (9), were used. For generation of these antibodies, fusion proteins from the NH2-terminal end of rat α-ENaC (amino acids E10-F77), from the COOH-terminal end of rat β-ENaC (amino acids G559-E636), and from the COOH-terminal end of rat γ-ENaC (amino acids A570-L650) were used (9, 10). Nitrocellulose membranes were blocked for 45 min at ambient temperature in 5% nonfat milk powder/Tris-HCl-buffered saline (TBS-T, 150 mM NaCl, 10 mM Tris-HCl, pH 8.5, 0.1% Tween 20) and exposed overnight at 4°C to anti-α-, β-, and γ-ENaC subunit antibodies diluted 1:1,000 in TBS-T. After washing in TBS-T, the membranes were exposed for 1 h at room temperature to the second antibody (peroxidase-labeled anti-rabbit Ig antibody, Amersham Life Sciences) diluted 1:7,500 in TBS-T. After washing in TBS-T, antigen-antibody complexes were detected by autoradiography by using enhanced chemiluminescence (ECL Western blotting analysis system, Amersham).

In Vivo Experiments

Adult male pigmented guinea pigs (300–400 g body wt, Elevage d’Ardenay, France) were anesthetized with intramuscular injection of 2 ml/kg body wt of a mixture of ketamine (50 mg/ml, Panpharma, Pougéres, France) and xylazine (2%, Rompun, Bayer, Leverkusen, Germany; 2:1, vol/vol). A heating table maintained the temperature between

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<tr>
<th>Table 1. Primers used for PCR of α-, β-, and γ-ENaC subunits</th>
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<td>PCR Product/Primer Sequence</td>
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<tr>
<td>α-ENaC: S: 5'-CTAGACCTCGCCCGCCTCCAC-3' AS: 5'-AGGTACTCTCAGCAACAGGCAAG-3'</td>
</tr>
<tr>
<td>β-ENaC: S: 5'-CAACACGCATATGCTCCACCC-3' AS: 5'-AGGCTAGGGAAATCATAGT-3'</td>
</tr>
<tr>
<td>γ-ENaC: S: 5'-ACAAAGACCTGAACCAAAAG-3' AS: 5'-GCACAGACAGGTAATAATG-3'</td>
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ENaC, epithelial Na channel; S, sense; AS, antisense.
36.5 and 37.5°C. The animal’s head was maintained in a ventral position by a head holder.

The bulla was opened through a lateral approach, and the cochlea was exposed. Glass micropipettes were bevelled to a tip diameter of 10–15 μm. The micropipette was inserted in a straight holder containing an Ag-AgCl pellet and endowed with a perfusion port (PC-S3 30° holder, Phymep, Paris, France). The holder was connected to an electrometer amplifier (604 differential electrometer, Keithley Instruments, Cleveland, OH). The perfusion port was coupled to a syringe with pump (341A, Sage Instrument, Cambridge, MA) with a polyethylene tube (external diameter: 0.96 mm; internal diameter: 0.58 mm; Polylabo, Strasbourg, France). The micropipette, holder, and polyethylene tube were filled with Na-rich (in mM: 125 NaCl, 25 NaHCO₃, 25 mannitol) or K-rich (in mM: 126 KCl, 1 NaCl, 25 KHC₂O₃, 25 mannitol) solutions. Immediately before injection, solutions were warmed to 37°C and gassed with 95% O₂ and 5% CO₂ so that their final pH was within the physiological range (7.35–7.45). An Ag-AgCl reference electrode was placed on the neck muscles. The micropipette was handled with a micromanipulator under stereomicroscopic observation. The tip of the micropipette was moved to the perilymph of the scala tympani through the round window and then to the endolymph of the scala media of the first cochlear turn through the basilar membrane (Fig. 1). The zero potential was determined in the perilymph of the scala tympani. Endocochlear potential of the first cochlear turn was recorded before, and 30 s, 1 min, 2 min, 3 min, and 15 min after the beginning of endolymphatic perfusion (100 nl/min for 3 min).

Normoxia experiments. During these experiments, animals breathed spontaneously. Five experimental series of perfusions of the scala media were performed: 1) K-rich solution (n = 11); 2) K-rich solution with 10⁻⁴ M amiloride (Sigma, St. Louis, MO) (n = 11); 3) Na-rich solution (n = 7); 4) Na-rich solution with 10⁻⁴ M amiloride (n = 5); and 5) Na-rich solution with 10⁻⁶ M benzamil (n = 4; Fluka, Buchs, Switzerland).

Hypoxia experiments. Animals were tracheotomized and artificially ventilated with a Harvard volumetric ventilator for small rodents. Tidal volume and ventilation frequency were 2 ml and 60 beats per min, respectively. One minute after the beginning of the 3-min endolymphatic perfusion, a 3-min hypoxia was induced by ventilating the animals with pure N₂. Evolution of the endocochlear potential was compared in two experimental conditions of K-rich solution perfusion in absence (n = 6) or presence of 10⁻⁶ M benzamil (n = 6).

Statistics

Results are expressed as means ± SE. Statistical significance between experimental groups was assessed by Student’s paired or unpaired t-test, with P < 0.05 considered significant.

RESULTS

RT-PCR

mRNA encoding α-, β-, and γ-ENaC subunits were detected by RT-PCR in microdissected cochlear soft tissues (Fig. 2). The sizes of amplified cDNA fragments are identical to those previously reported in the literature with the same PCR primers (20, 29). No contamination with genomic DNA was observed when RT-negative controls were amplified by PCR.

In Situ Hybridization

Negative controls with sense probes showed quite a high background signal because of the long exposition in the photoemulsion (6 wk) (Fig. 3). With α-ENaC mRNA-specific probe, a significant signal was observed in epithelia that border endolymph, i.e., the stria vascularis, spiral prominence, and spiral limbus, as well as in two nonepithelial tissues, the inferior part of the spiral ligament, and the spiral ganglion. The signals obtained with β- and γ-subunit-specific probes were weaker. A significant signal was found for the β-subunit in the stria vascularis, spiral prominence, spiral limbus, and spiral ligament, and for the γ-subunit in spiral limbus, spiral ligament, and spiral ganglion.

Western Blot

The three ENaC subunit proteins were detected by Western blot in cochlear soft tissues with similar MW to the ones previously reported in mouse and rat kidney cortical collecting duct cell lines (Fig. 4) (3, 9).

![Fig. 2. Detection of epithelial Na channel (ENaC) α-, β-, and γ-subunits mRNA by RT-PCR in the rat cochlea. After 40 cycles of PCR on RT products, a single band was visualized for α-, β-, and γ-subunits mRNA at the expected sizes (RT+). No signal was observed when the RT was omitted during the reverse transcription (RT-). Base pairs (bp): DNA size marker.](http://ajprenal.physiology.org/Downloadedfrom/10.1152/ajprenal.00155.2003)
In Vivo Experiments

Initial endocochlear potential and mechanical effect of endolymphatic perfusion. Initial endocochlear potential values of the different experimental groups were not statistically different (one-way ANOVA, \( P = 0.15 \)). Nevertheless, these values were about 18 mV higher before K-rich solution perfusion (184 ± 2.7 mV, \( n = 11 \), and 183 ± 3.8 mV, \( n = 11 \), in control and amiloride groups, respectively; Fig. 5) than before Na-rich solution perfusion (175 ± 2.9 mV, \( n = 7 \), 173 ± 4.9 mV, \( n = 5 \), and 180 ± 2.3 mV, \( n = 4 \), in control, amiloride, and benzamil groups, respectively; Fig. 6) or before K-rich solution perfusion in artificially ventilated animals (174 ± 5.0 mV, \( n = 6 \), and 175 ± 3.5 mV, \( n = 6 \), in control and benzamil groups, respectively; Fig. 7). Values obtained before K-rich solution perfusion under normoxic conditions were similar to the ones usually obtained before K-rich solution perfusion under hypoxic conditions but were significantly higher than those obtained before Na-rich solution perfusion under hypoxic conditions (175 ± 2.9 mV, \( n = 7 \), 173 ± 4.9 mV, \( n = 5 \), and 180 ± 2.3 mV, \( n = 4 \), in control, amiloride, and benzamil groups, respectively; Fig. 6).

Fig. 3. Localization of \( \alpha \)-(A-C), \( \beta \)-(D-F), and \( \gamma \)-ENaC subunit (G-I) mRNA by in situ hybridization in the rat cochlea. Midmodiolar sections of cochlear, second turn. A, D, G: light microscopy; B, E, H: dark-field antisense probe; C, F, I: dark-field sense probe. In the negative controls (sense probes: C, F, I), the background signal was quite elevated due to the long duration (6 wk) of exposition in photoemulsion. With the antisense probe (B, E, H), a specific signal for \( \alpha \)-ENaC subunit mRNA was observed in stria vascularis, spiral prominence, spiral limbus, spiral ligament, and spiral ganglion. \( \beta \)-Subunit mRNA was detected in all those tissues except the spiral ganglion. \( \gamma \)-Subunit was present in spiral limbus, spiral ligament, and spiral ganglion, but not in stria vascularis and spiral prominence.

Fig. 4. Detection of \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC subunit proteins by Western blot in the rat cochlea. \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC subunit proteins were detected by Western blot with rabbit polyclonal antibodies (10). Specificity, as well as migration pattern, were previously described (3, 9). MW: in kDa.
reported at the first turn of guinea pig cochlea (35). In the two other groups, the relatively low endocochlear potential values could have resulted from a small Na leak out of the microelectrode into endolymph before the beginning of the Na-rich perfusion, and from a small hypoxia- and/or stress-related hormone release related to tracheotomy and artificial ventilation before K-rich solution perfusion under hypoxic conditions.

During the endolymphatic perfusion, a small and transient increase in the endocochlear potential was observed, whatever the composition of the solution (Fig. 5–7). This increase was previously reported and ascribed to mechanical alteration of the structure of the scala media during perfusion (17).

Lack of effect of ENaC inhibitors under normoxic conditions. When K-rich solution was perfused, endocochlear potential was not altered at 15 min compared with the beginning of the experiment (Fig. 5) \(P > 0.05\), paired Student’s \(t\)-test). Amiloride did not modify the evolution of the EP under this experimental condition (Fig. 5).

When Na-rich solution was perfused, the endocochlear potential value progressively fell after the end of the perfusion, reaching 34\% \(\pm\) 3.9 (\(n = 7\)) of its initial value 15 min after the beginning of the perfusion. Amiloride and benzamil, in Na-rich solution, rapidly increased the endocochlear potential as soon as 30 s, and up to 3 min, and 15 min after the beginning of the perfusion, respectively (Fig. 6). This rapid effect suggests that ENaC was functionally active at the apical membrane of epithelial cells facing endolymph.

Effect of ENaC inhibitors under hypoxic conditions. As previously reported (5), hypoxia induced a rapid endocochlear potential decrease that was maximal at the end of hypoxia, i.e., 4 min after the beginning of the experiment (Fig. 7). At that time, endocochlear potential was 15\% \(\pm\) 6.0 (\(n = 6\)) of the initial value (endocochlear potential at 1 min: \(179 \pm 6.2\) mV; endocochlear potential at 4 min: \(110 \pm 4.5\) mV, \(n = 6\)). The addition of benzamil in the perfused K-rich solution did not modify the endocochlear potential evolution (19\% \(\pm\) 7.0 of the initial value at 4 min, \(n = 6\), Fig. 7).

**DISCUSSION**

The purpose of the present study was to check the presence and to assess the role of ENaC in the cochlea. ENaC \(\alpha\), \(\beta\), and \(\gamma\)-subunits were detected in rat cochlea both at the mRNA (RT-PCR) and protein (West-
ern blot) levels. ENaC mRNA was localized by in situ hybridization in several epithelial (stria vascularis, spiral prominence, spiral limbus) and nonepithelial (spiral ligament, spiral ganglion) structures of rat cochlea. This Na channel may be involved in the maintenance of the Na chemical gradient between Na-poor endolymph (1 mM) and Na-rich perilymph (140 mM). Thus the in vivo function of this channel likely is to contribute to Na transport out of endolymph to maintain a very low endolymphatic Na concentration.

Presence of ENaC in Epithelial and Nonepithelial Rat Cochlear Tissues

ENaC mRNA was localized by in situ hybridization in several epithelial and nonepithelial tissues. The labeled epithelial structures, stria vascularis, spiral prominence, and spiral limbus, border endolymph. In these epithelia, ENaC is probably involved in Na trans-epithelial vectorial transport resulting in Na extrusion from endolymph.

ENaC mRNA was also localized in two nonepithelial cochlear tissues, the spiral ligament, and the spiral ganglion. This channel has been found in several nonepithelial cells such as keratinocytes (6), lymphocytes (28), eye endothelial cells, fibroblasts, and neurons (photoreceptors, bipolar cells, ganglia) (26). The functions of ENaC in nonepithelial cells probably depend on the cell type and remain to be elucidated. In spiral ligament fibrocytes, ENaC may be more specifically involved in vectorial ion transports. This structure has been proposed to be involved in the recycling of K from the organ of Corti to the stria vascularis (4, 32, 34). As a matter of fact, these cells are endowed with tight junctions and with several Na and K transport systems (Na-K-Cl cotransport, Na\(^+/\)K\(^+\)-ATPase, Na\(^+/\)H\(^+\) exchanger). ENaC might regulate the K recycling pathway by modifying the resting potential and intracellular Na concentration of spiral ligament fibrocytes.

The observation of positive signals for ENaC subunits mRNA in the cochlear spiral ganglion is in accordance with recent works in which ENaC was detected in nervous structures such as eye photoreceptors, bipolar cells, and ganglia (26). The role of ENaC in these structures is unclear. As reported for Phe-Met-Arg-Phe-amide-activated Na channel, another member of the degenerin/ENaC superfamily (22), ENaC may be functionally coupled to neurotransmission processes.

Concerning the stoichiometry of ENaC subunits, the presence of the three subunits of ENaC in the rat cochlea was attested to at both the mRNA and protein levels by RT-PCR and Western blot, respectively. In situ hybridization clearly showed that the α-subunit mRNA was expressed in several epithelial and nonepithelial structures of the cochlea. However, because of the low signal intensity obtained with β- and γ-subunit mRNA-specific probes in the present study, further work is needed to assess the exact distribution of β- or γ-subunits of ENaC in the cochlea. Additional information concerning the topography of ENaC-subunits could be brought at the protein level by immunohistochemistry. Note that this does not preclude the existence of a functional Na channel in those tissues because of the expression of α-subunit in *Xenopus laevis* oocytes was sufficient to give rise to an amiloride-sensitive Na current, and the addition of β- or γ-subunits increased the activity of the Na channel (7).

Role of ENaC in the Regulation of Na Endolymph Concentration

The Na electrochemical gradient that exists between Na-poor endolymph and Na-rich perilymph in physiological conditions indicates that active Na absorption from endolymph is achieved by epithelia lining these compartments. The observation of ENaC mRNA in some of these epithelia, namely the stria vascularis, spiral prominence, and spiral limbus, suggests that ENaC probably contributes to this active Na resorption. Other apical Na transport systems such as non-selective cationic channels in outer sulcus cells (23), apical transduction channels in hair cells (25), or NHE3 isoform of the Na\(^+\)/H\(^+\) exchanger in marginal cells (4) may also contribute to Na extrusion from endolymph. The lack of effect of amiloride (present study, Fig. 5; 33) and benzamil (Fig. 7) on the endocochlear potential in K-rich endolymph suggests that ENaC activity is not a major determinant of the endocochlear potential. When the scala media was perfused with a Na-rich solution, ENaC activity became, as expected, a determinant of the endocochlear potential, as shown by the endocochlear potential increase induced by the luminal application of amiloride and benzamil (Fig. 6). This result confirms that ENaC is located in the apical membrane of epithelia facing endolymph, and is consistent with previous observations (12, 38). Indeed, recording of short circuit current in stria vascularis marginal cell line mounted into Ussing chamber showed an amiloride-sensitive transepithelial Na flux (38). Similarly, a transepithelial Na flux inhibited by amiloride has also been observed in in vitro preparations of frog semicircular canal ampulla (12), an inner ear structure in which epithelial transport systems are quite similar to those observed in the cochlea (39).

It must be emphasized that the effects of amiloride and benzamil in Na-rich solutions cannot be due to the inhibition of hair cell nonselective cationic transduction channels (31), because no endocochlear potential increase was observed when amiloride was administered in K-rich solutions (present study, 33). Neither did the endocochlear potential increase result from the inhibition of apical Na\(^+\)/H\(^+\) exchanger, because this electroneutral antiporter is not inhibited by benzamil at the low concentration used in the present study (final concentration < 10\(^{-6}\) M) (18). Thus the endocochlear potential increase induced by amiloride and benzamil during Na-rich perfusions presumably resulted from the inhibition of Na reabsorption from endolymph through apical ENaC.

Physiologically, endolymph is a Na-rich fluid during the fetal period. During the ultimate phase of inner ear
functional maturation, i.e., at the end of gestation for
the guinea pig, or during the first week of life in mice
and rats, endolymph becomes a Na-poor and K-rich
fluid (1, 30). ENaC could be involved in the clearance of
endolymhatic Na during this shift in the composition of
endolymph. This role of ENaC in the inner ear can be
paralleled with the role of ENaC in lung maturation,
during which ENaC participates in the clearance of the
fluid that fills newborn airways (16). When the K-rich
and Na-poor composition of endolymph is settled, the
function of ENaC remains necessary to clear the Na
that reaches the endolympathic compartment by para-
cellular passive leak.

In pathophysiology, an increase in endolymhatic Na
concentration has been described during anoxia,
from 3–6 to 32 mM, after 2- and 32-min anoxia, respec-
tively (5). In the present study, administration of ben-
zamil in the scala media did not alter the evolution of
the endocochlear potential during and after a 3-min
period of hypoxia (Fig. 6). This may be due to the fact
that the modification of Na concentration (approxi-
mately or equal to +6 mM) induced by this short period
of hypoxia was insufficient to significantly influence
the endocochlear potential. Endolymhatic Na concen-
tration may also be increased during endolympathic
hydrops, which is an increase in endolymph volume.
Endolymhatic hydrops constitutes the anatomic sub-
stratum of Menière’s disease, a syndrome character-
ized by attacks of vertigo, sensorineural hearing loss,
and tinnitus. Attacks of vertigo during this pathology
have been ascribed to the sudden disruption of dis-
tended Reissner’s membrane (25) with subsequent con-
tamination of endolymph by Na. In this pathology, ENaC
may participate in the clearance of Na out of
endolymph.

In conclusion, all three ENaC subunit mRNAs and
proteins were detected in rat cochlea. ENaC mRNA
was localized in situ hybridization in both epithelial
and nonepithelial cochlear structures. The rapid endo-
cochlear potential increase induced by endolympathic
perfusion of ENaC inhibitors, amiloride and benzamil,
dissolved in Na-rich solution, indicates that ENaC is
functionally active in the apical membrane of epithelia
facing endolymph, stria vascularis, spiral prominence,
and spiral limbus. This strongly suggests that ENaC
transport activity contributes to maintenance of a very
low Na concentration in endolymph.

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