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ARTICLE

The Detailed Localization Pattern of $Na^+/K^+/2Cl^-$ Cotransporter Type 2 and Its Related Ion Transport System in the Rat Endolymphatic Sac

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SUMMARY The endolymphatic sac (ES) is a part of the membranous labyrinth. ES is believed to perform endolymph absorption, which is dependent on several ion transporters, including $Na^+/K^+/2Cl^-$ cotransporter type 2 (NKCC-2) and Na^+/K^+-ATPase. NKCC-2 is typically recognized as a kidney-specific ion transporter expressed in the apical membrane of the absorptive epithelium. NKCC-2 expression has been confirmed only in the rat and human ES other than the kidney, but the detailed localization features of NKCC-2 have not been investigated in the ES. Thus, we evaluated the specific site expressing NKCC-2 by immunohistochemical assessment. NKCC-2 expression was most frequently seen in the intermediate portion of the ES, where NKCC-2 is believed to play an important role in endolymph absorption. In addition, NKCC-2 expression was also observed on the apical membranes of ES epithelial cells, and Na^+/K^+-ATPase coexpression was observed on the basolateral membranes of ES epithelial cells. These results suggest that NKCC-2 performs an important role in endolymph absorption and that NKCC-2 in apical membranes and Na^+/K^+-ATPase in basolateral membranes work coordinately in the ES in a manner similar to that in renal tubules. **(J Histochem Cytochem 58:759–763, 2010)**

KEY WORDS
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Na^+/K^+-ATPase
endolymph
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THE ENDOLYMPHATIC SAC (ES) is a part of the membranous labyrinth that contains the cochlea, vestibular organs, and semicircular canals. It is accepted that ES has several possible roles; in particular, endolymph absorption is the main function of the ES because dysfunction of endolymph by obstruction of the endolymphatic duct induced endolymphatic hydrops, a characteristic pathological finding of Meniere's disease (Kimura and Schuknecht 1965; Lundquist 1965); therefore, regulation of endolymph by the ES is fundamental to the maintenance of endolymph homeostasis. In accordance with a report by Guild (1927), the ES was subdivided into three parts, proximal sac portion (PSP), intermediate sac portion (ISP), and distal sac portion (DSP), and this proposed subdivision is still being followed. Although absolute confirmation has not yet been obtained, ISP may be the most important part of the ES involved in endolymph absorption (Hoshikawa et al. 1994; Dahlmann and von During 1995; Miyashita et al. 2007).

Endolymph absorption may depend on transport of ions, such as Na^+ and Cl^-, which may be the dominant ions in the ES (Amano et al. 1983; Mori et al. 1987). The existence of several ion channels, such as epithelial Na^+ channel (Mori and Wu 1996) and nonselective cation channel (Miyashita et al. 2001), and transporters, such as Na^+/K^+-ATPase (Yamane and Nakai 1988), $Na^+/K^+/2Cl^-$ cotransporter type 2 (NKCC-2; Akiyama et al. 2007), and thiazide-sensitive Na^+/Cl^- cotransporter (Akiyama et al. 2008), in the ES epithelium has been reported. In particular, Na^+/K^+-ATPase is considered to be an important ion transporter

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producing a driving force for ion transport (Miyashita et al. 2007). NKCC-2 is also an ion cotransporter expressed in the ES epithelium (Akiyama et al. 2007). NKCC-2 is typically recognized as a kidney-specific ion transporter and is expressed in the apical membrane of the thick ascending limb (TAL) loop of Henle, where it is responsible for the reabsorption of ~20% of filtered NaCl (Gamba et al. 1994). Recently, the protein expression of NKCC-2 in human and rat ES has been reported by using IHC (Nishimura et al. 2008; Kakigi et al. 2009); however, detailed tissue or cellular localization has not been reported.

This study has elucidated the NKCC-2 localization pattern in each part of the ES and the coexpression pattern with Na^+/K^+-ATPase by immunohistochemical assessment, which may provide beneficial information to understand the ES ion transport system.

Materials and Methods

Animals

Four-week-old Sprague–Dawley rats were purchased from Charles River Japan (Yokohama, Japan) and were used for all experiments. This research was approved by the Animal Care and Use Committees of Kagawa University.

Antibodies

Antibodies used in this study were as follows: primary antibodies, polyclonal rabbit anti-rat NKCC-2 (1:100 dilution; Millipore, Billerica, MA) and monoclonal mouse anti-rat Na^+/K^+-ATPase α-1 subunit (1:200 dilution; Millipore); secondary antibodies, Alexa Fluor 488–conjugated goat anti-rabbit IgG and Alexa Fluor 546–conjugated goat anti-mouse IgG (1:100 dilution; Invitrogen, Carlsbad, CA).

Immunofluorescence Double Staining

Tissue preparation and immunostaining were performed as described in our previous study with minor modifications (Akiyama et al. 2008). Briefly, rats were deeply anesthetized with ketamine hydrochloride (50 mg/kg IM.; Daiichi-Sankyo, Tokyo, Japan) and diethyl ether, exsanguinated via the left ventricle with a 4% paraformaldehyde (PFA) mixture in PBS for ~5 min, and then decapitated. The temporal bones were rapidly removed, and the ESs on bilateral sides, including the surrounding bone tissues, were dissected carefully under stereomicroscope (LEICA MZ125; Leica Microsystems, Tokyo, Japan). The whole kidney was also dissected for the control study. The ESs and kidneys were fixed in 4% PFA in PBS overnight at 4C, and the ESs were decalcified in 0.12 M EDTA (pH 6.5) for ~10 days at 4C. The EDTA solution was changed daily. After overnight treatment in 30% sucrose, the decalcified ESs and kidneys were embedded in OCT tissue compound (Sakura Finetek; Tokyo, Japan), and 5-μm-thick sections were made using cryostat at −20C. The sections were washed with distilled water and PBS and were covered with blocking reagent (Nichirei Bio; Tokyo, Japan) for 30 min to block unspecific binding sites. They were then covered with anti-NKCC-2– and anti-Na^+/K^+-ATPase–specific primary antibodies for overnight incubation at 4C. After repeated washing with PBS containing 0.2% Tween 20, the sections were incubated with secondary antibodies for 2 h at room temperature to detect the binding sites of primary antibodies. After repeated washing again with PBS containing 0.2% Tween 20, the sections were mounted using SlowFade Gold (Invitrogen) on a slide, and the slides were observed under an OLYMPUS BX51 light microscope (Olympus; Tokyo, Japan). The appropriate excitation wavelength and emission filter wavelength for Alexa Fluor 488 are 470–490 and 510–550 nm; for Alexa Fluor 546 they are 520–550 and 580 nm, respectively. Images were taken at a magnification of 100× (objective: 10 × 0.4) and 200× (objective: 20 × 0.7). Negative control experiments, in which the primary antibodies were omitted, were performed using kidney and ES sections. To confirm the accuracy and reproducibility, five rats (10 sides of ES) were included in this immunofluorescent study.

Results

Rat ES sections were divided into three parts in accordance with the previous anatomic studies (Guild 1927; Dahlmann and von During 1995). PSP is situated in the intraosseous part, and most of the epithelial cells in PSP consisted of short and thin squamous cells. The proximal side of the ISP is located in the intraosseous part and the distal side is in the extraosseous part. DSP is in the extraosseous part, its lumen is sometimes collapsed, and it lies between the transverse sinus and the subarachnoid space. Its lumen is covered with smooth and short squamous cells. These findings are in accordance with the previous reports (Dahlmann and von During 1995). Figures 1A–1C show the anatomic location of the PSP, ISP, and DSP with surrounding temporal bone tissues, respectively.

The sections of kidney and ES separated from each part were double stained with anti-NKCC-2– and anti-Na^+/K^+-ATPase–specific primary antibodies (Figure 2A). Green and red signals indicate NKCC-2– and Na^+/K^+-ATPase–immunoreactive regions, respectively. NKCC-2–immunopositive signals were observed on the apical membrane side of the renal tubules at the renal medulla (except for inner medulla) and renal cortex, which is in agreement with the typical NKCC-2 expression pattern reported previously (Ecelbarger et al. 1996). NKCC-2 signals were specifically observed on

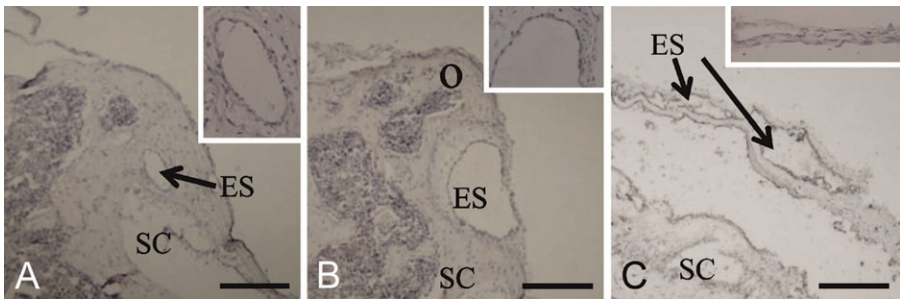

Figure 1 Serial sections of rat ES from PSP to DSP stained with hematoxylin. **A**, **B**, and **C** show PSP, ISP, and DSP, respectively. Each right upper inset is the high-power field of ES epithelium. ES, endolymphatic sac; PSP, proximal sac portion; ISP, intermediate sac portion; DSP, distal sac portion; SC, semicircular canal; O, operculum. Bar = 200 μm.

the apical membrane side of the ES epithelia, similar to the expression pattern observed in the kidney epithelia, and Na^+/K^+-ATPase signals were specifically observed on the basolateral membrane side, which again are in agreement with the NKCC-2 expression pattern reported previously (ten Cate et al. 1994). When comparing PSP, DSP, and ISP, NKCC-2 and Na^+/K^+-ATPase showed the highest intensity level at ISP. The intensity levels of NKCC-2 and Na^+/K^+-ATPase at PSP were lower than those at ISP. Na^+/K^+-ATPase signals were observed in all ES regions; in contrast, almost no immunopositive signal of NKCC-2 was observed in DSP. No positive signal was observed in sections without primary antibodies.

NKCC-2– and Na^+/K^+-ATPase–positive signals were localized on apical and basolateral membranes, respectively. The merged images in Figure 2 indicate their expression pattern more clearly and demonstrate that almost all NKCC-2–positive cells are coexpressed with Na^+/K^+-ATPase.

Discussion

The electrolyte composition of the endolymph in the ES is significantly different from that in the cochlea and vestibule. The endolymph in the cochlea and vestibule is rich in K^+ and poor in Na^+, whereas that in the ES is rich in Na^+ and poor in K^+ (Mori et al. 1987; Couloigner et al. 1999). In addition, Na^+ and Cl^- outflow from the lumen of the ES to the extracellular space was reported (Amano et al. 1983; Mori et al. 1987), and surgical obliteration of the ES induces endolymphatic hydrops (Kimura and Schuknecht 1965; Swart and Schuknecht 1988), suggesting that ES may have important roles in endolymph absorption via specific Na^+- and Cl^--regulatory mechanisms.

NKCC-2 is an electroneutral cotransporter with a stoichiometry of $1Na^+:1K^+:2Cl^-$ and is known to function in an absorptive manner. NKCC-2 was originally known as a kidney-specific cotransporter expressed only on the apical membrane of the TAL (Knepper and Brooks 2001; Mutig et al. 2007). Its expression has been reported recently in human and rat ES (Nishimura et al. 2008; Kakigi et al. 2009); however, its detailed localization has not been reported. The present immunostaining results with specific anti-NKCC-2 antibody indicate that NKCC-2 is localized in the apical membranes of ES epithelia, specifically in the same pattern as in mammalian renal TAL (Knepper and Brooks 2001). The cellular localization pattern suggests that NKCC-2 is concerned with Na^+ and Cl^- influx from the lumen of the ES to the intracellular space and accompanied water absorption, and it emphasizes the characterization that ES epithelia have an absorptive function. Water may be transported through the aquaporin (AQP) family, and the actual expressions of several AQPs in the ES were reported (Beitz et al. 1999; Nishimura et al. 2008; Kakigi et al. 2009). In the kidney, ion transporters (including NKCC-2 and Na^+/K^+-ATPase) produce an osmotic gradient, which becomes the driving force of water flux via AQPs. A similar water absorption system may exist in the ES, but this hypothesis is preliminary.

In addition, the strongest NKCC-2–positive signals were observed at ISP, and comparatively weak signals were observed at PSP; conversely, DSP had fewer NKCC-2–positive signals. It has been reported that ISP has an important role in the absorption of endolymph, and PSP is also suggested to have some degree of absorptive ability (Dahlmann and von During 1995). DSP is believed to have little correlation with the absorption of endolymph. In the kidney, NKCC-2 is expressed in the cortical TAL, medullary TAL, and macula densa cells. Some physiological studies have characterized a number of differences in salt transport and affinity of three ions in cortical TAL and medullary TAL that may be related to the differences in the NKCC-2 expression level or splicing variant isoform expression pattern. Medullary TAL has high transport capacity, but cortical TAL has a lower transport rate. Using immunoblotting and IHC, it has been reported that much stronger NKCC-2 expression was observed in the medulla than in the cortex (Ecelbarger et al. 1996). Results of this study and previous studies indicate that the strongest NKCC-2 expression was observed at ISP, which is the central location for ion transport in the ES. We therefore speculate that the degree of NKCC-2 expression may reflect the absorptive function

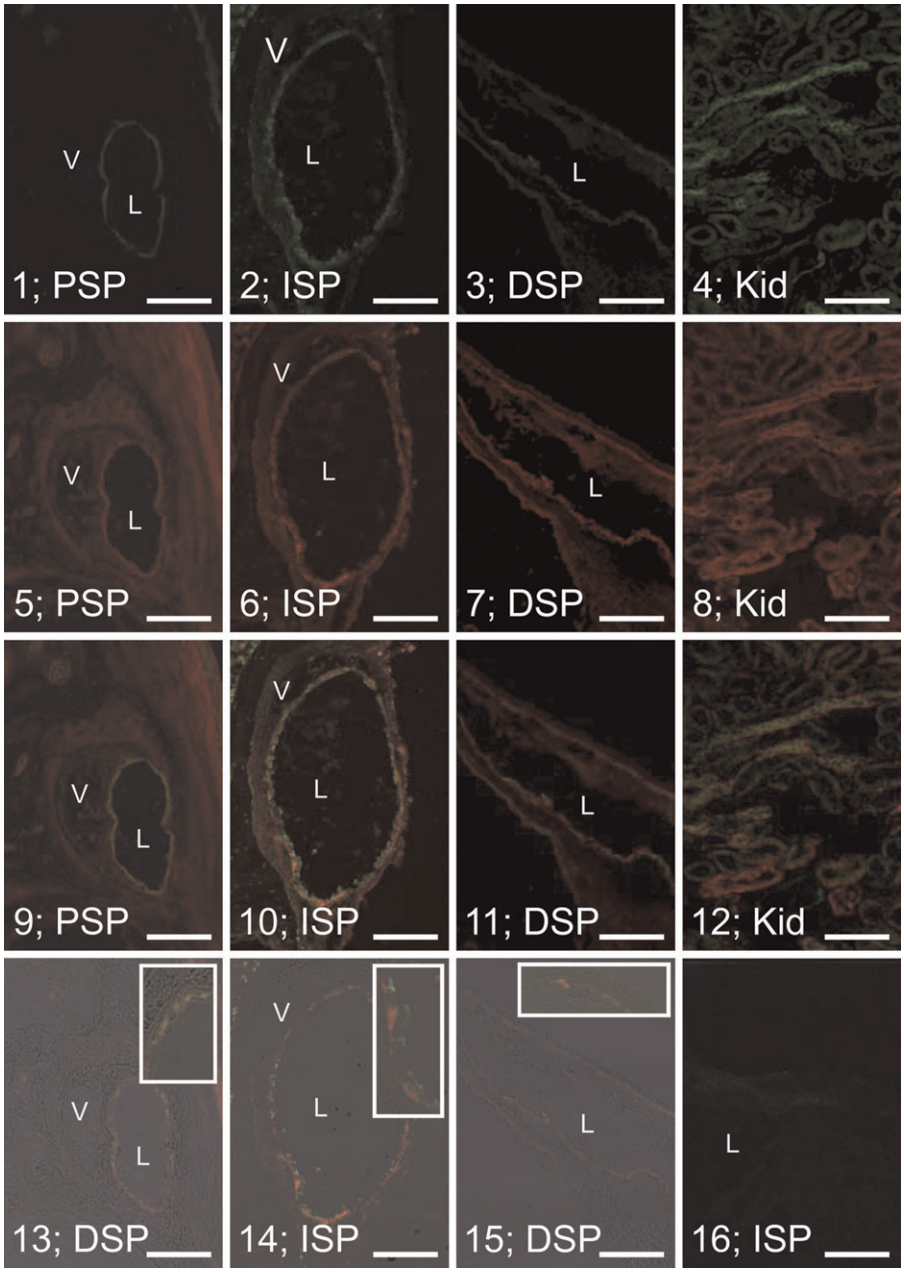

Figure 2 Immunofluorescent double staining with specific antibodies for anti-$Na^+/K^+/2Cl^-$ cotransporter type 2 (anti-NKCC-2) and anti-Na^+/K^+-ATPase. Green and red signals indicate NKCC-2– and Na^+/K^+-ATPase–immunoreactive cells, respectively. NKCC-2 (1–3): apical membranes of ES epithelia are locally stained; positive cells are most frequently observed at ISP, weaker positive signals are observed at PSP, and almost no signal is observed at DSP. Na^+/K^+-ATPase (5–7): basolateral membranes of ES epithelia are locally stained; positive cells are observed in all ES individual parts, but the positive region at PSP is more partial than that at ISP, and DSP shows faint staining. Kidney is shown as a positive control (4 and 8). Merged images are also shown to indicate the coexpression pattern of NKCC-2 with Na^+/K^+-ATPase (dark field: 9–12 and bright field: 13–15). Right upper insets in 13–15 show the high-power field of ES epithelium. As a negative control, ISP merged image without primary antibodies is also shown, and no labeling was observed (16). Labeling conditions and microscope settings were identical for all ES sections. PSP, proximal sac portion; ISP, intermediate sac portion; DSP, distal sac portion; Kid, kidney; L, lumen of the ES; V, vein of vestibular aqueduct. Bar = 50 μm.

in each part of the ES, similar to that in the kidney. These results suggest that NKCC-2 plays critical and fundamental roles in ES ion transport.

Additionally, we performed double staining using antibodies specific to anti-NKCC-2 and anti-Na^+/K^+-ATPase to evaluate their coexpression pattern. Na^+/K^+-ATPase was composed of an α subunit and a β subunit. The α subunit contains the binding sites for Na^+, K^+, and ATP, and the β subunit incorporates the α subunit into the cell membrane (Blanco and Mercer 1998; Hasler et al. 1998). In this study, we performed immunostaining with a specific monoclonal antibody for the α-1 subunit, which appears to be the major or single α subunit to be expressed along the nephron and is the predominant subtype at the ES epithelium (ten Cate et al. 1994). Na^+/K^+-ATPase is known to be expressed in the basolateral membranes throughout the renal tubules and to also function in active Na^+ transport. Especially at the TAL, it works as one unit linked with NKCC-2 and several channels and provides the driving force for subsequent Na^+ influx from the apical membrane side, mainly through NKCC-2 (Brenner 2000). In the ES, the positive signals were observed ubiquitously on the basolateral

membrane side of epithelial cells, as in previous studies (Mizukoshi et al. 1988). Almost all NKCC-2–positive cells showed positive staining for Na^+/K^+-ATPase, suggesting that in the ES, NKCC-2 may act coordinately with Na^+/K^+-ATPase in a manner similar to that in kidney TAL. Na^+/K^+-ATPase, which is distributed throughout the basolateral membranes of epithelial cells, and NKCC-2, which is distributed in the apical membranes of epithelial cells, are well-known as the main Na^+ and K^+ (Cl^-) transport pathways in renal tubules (Brenner 2000). Likewise, they may play a major role in mammalian ES.

In conclusion, we confirmed that NKCC-2, which was localized on the apical membrane side of the ES in a typical kidney expression pattern, was expressed more strongly in ISP than in PSP and DSP and that, in addition, NKCC-2 was coexpressed with Na^+/K^+-ATPase. These data suggest that NKCC-2 and Na^+/K^+-ATPase contribute to the Na^+ absorption from the apical or basolateral membrane side in cooperation with each other in the ES epithelial cells.

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