http://dx.doi.org/10.5607/en.2013.22.4.322
Exp Neurobiol. 2013 Dec;22(4):322-329.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

en
Experimental Neurobiology

Developmental Role of Anoctamin-1/TMEM16A in Ca^{2+}-Dependent Volume Change in Supporting Cells of the Mouse Cochlea

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Mammalian cochlea undergoes morphological and functional changes during the postnatal period, around the hearing onset. Major changes during the initial 2 postnatal weeks of mouse include maturation of sensory hair cells and supporting cells, and acquisition of afferent and efferent innervations. During this period, supporting cells in the greater epithelial ridge (GER) of the cochlea exhibit spontaneous and periodic activities which involves ATP, increase in intracellular Ca^{2+}, and cell volume change. This Ca^{2+}-dependent volume change has been proposed to involve chloride channels or transporters. We found that the spontaneous volume changes were eliminated by anion channel blocker, 100 μM NPPB. Among candidates, expression of Anoctamin-1 (Ano1 or TMEM16A), bestriphin-1 and NKCC1 were investigated in whole-mount cochlea of P9-10 mice. Immunolabeling indicated high level of Ano1 expression in the GER, but not of betrophin-1 or NKCC1. Double-labeling with calretinin and confocal image analysis further elucidated the cellular localization of Ano1 immunoreactivity in supporting cells. It was tested if the Ano1 expression exhibits similar time course to the spontaneous activities in postnatal cochlear supporting cells. Cochlear preparations from P2-3, P5-6, P9-10, P15-16 mice were subjected to immunolabeling. High level of Ano1 immunoreactivity was observed in the GER of P2-3, P5-6, P9-10 cochleae, but not of P15-17 cochleae. Taken together, the localization and time course in Ano1 expression pattern correlates with the spontaneous, periodic volume changes recorded in postnatal cochlear supporting cells. From these results we propose that Ano1 is the pacemaker of spontaneous activities in postnatal cochlea.

Key words: Anoctamin-1 (Ano1)/TMEM16A, cochlea, greater epithelial ridge, cell volume change, Ca^{2+}-activated chloride channel, supporting cell

INTRODUCTION

The sensory epithelium of the developing cochlea can be divided into 2 anatomical regions, the greater epithelial ridge (GER) and lesser epithelial ridge (LER). The GER refers to the area medial to pillar cells, consisting inner hair cells (IHCs) and inner supporting cells. The LER spans the area radial to pillar cells, consisting outer hair cells (OHCs) and outer supporting cells.

Received December 9, 2013, Revised December 14, 2013,
Accepted December 14, 2013

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Most types of cells in the GER and LER of the mouse cochlea undergo dramatic functional, neural, and morphologic changes during the initial 2 postnatal weeks. Sensory hair cells mature from action potential firing immature cells to graded-depolarization generating adult forms [1]. Auditory afferent nerve fibers continue to reorganize their dendritic structures and acquire faster-gating, adult type glutamate receptors [2, 3]. Auditory efferent nerve fibers innervating sensory hair cells undergo pronounced changes as well. Transient formation and loss of auditory efferent synapses onto inner hair cells and gradual acquisition of efferent nerve fiber-driven electronmotility in outer hair cells have been reported [4-6].

Changes in non-sensory supporting cells are even more dramatic. The GER supporting cells exhibit periodic spontaneous activities [7]. The spontaneous activities of supporting cells were present from birth until shortly after the hearing onset. Lines of evidence have indicated that the spontaneous activities in the developing cochlea are related to ATP-mediated signaling. After ATP is released through connexin hemichannels of a supporting cell, it activates P2X and P2Y receptors in adjacent inner hair cells and supporting cells, increases intracellular Ca^{2+} concentration in these cells, and further spreads the signal to surrounding cells until ATP is degraded by extracellular nucleotidases [7-9]. The supporting cell initiated-spontaneous activities cause bursts of action potentials in the inner hair cells and auditory afferent nerve fibers [7, 10]. It is thought that such burst of action potentials in the auditory afferent nerve fibers help tonotopic wiring of the hearing end organ and the auditory information processing counterpart in the brain before hearing onset.

Somewhat similar ATP-mediated signals were observed in the LER supporting cells as well [11, 12]. Indeed, ATP-mediated signaling in both GER and LER areas shares much of the same molecular players, such as connexin hemichannels, P2X receptors, P2Y receptor coupled IP_3 generation, and IP_3-induced Ca^{2+} release from intracellular stores [13-15].

Despite such similarities, ATP-mediated signaling in GER and LER exhibited clear differences. Firstly, ATP-mediated Ca^{2+} waves in LER are rarely spontaneous [12]. Secondly, the spontaneous activities in the GER supporting cells accompany very peculiar and noticeable cell shrinkage [7, 8]. Exogenously applied ATP also causes similar sequential ATP-mediated signals and cell shape changes in GER supporting cells. On the other hand, ATP-mediated signals in LER do not exhibit any noticeable such change [11, 13, 14].

The molecular player(s) enabling spontaneous ATP release and cell volume change in GER supporting cells is not fully identified. Some evidence suggested that the cell volume change in GER may be due to water secretion accompanying Ca^{2+}-activated chloride flux [8]. The cell volume changes are always coincided with increased intracellular $[Ca^{2+}]$ [7, 8]. Photo-liberation of caged Ca^{2+} caused supporting cell volume change similar to the spontaneous one. Apparent inward current from the baseline was recorded during whole-cell patch-clamp recording of Ca^{2+} uncaging experiment (which indicates either cation influx or anion outflux). Moreover, the spontaneous cell volume change in GER was blocked by DIDS, a chloride channel blocker. However, a controversy still remains because slow-phase of ATP-mediated signaling in LER supporting cells is also inhibited by DIDS [11]. Considering relative lack of selectivity of DIDS among different chloride channels it is interesting to find out if different subtypes of Ca^{2+}-activated chloride channels are expressed in supporting cells of GER and LER.

Here, mouse cochleae before and after hearing onset were examined by immunofluorescence labeling and confocal microscopy techniques. The cellular localization and time-course of expression pattern are compared with the spontaneous activities in the GER of developing cochlea.

MATERIALS AND METHODS

C57BL6 mice of either sex were used. All animal handling procedures were performed in accordance with institutional guidelines of KIST (Seoul, Korea). Inner ears were quickly removed from P2-3, P5-6, P9-10 and P15-17 C57BL6 mice, perfused through the round and oval windows with cold 4% paraformaldehyde prepared in PBS (pH7.4) and then fixed for 1 h at 4°C. The preparations were washed 3 times in PBS. Bone encapsulating the cochlear coil was carefully chipped away and the cochlear segments containing the organ of Corti were excised. After 1h blocking (room temperature, in solution containing 5% of either normal goat serum or donkey serum according to the secondary antibodies used, 0.25% Triton X-100 and PBS) the samples were incubated with primary antibodies diluted in blocking solution (overnight at 4°C). After three washes of 15 min in PBS, the samples were incubated with fluorescence labeled secondary antibodies diluted in blocking buffer (for 1 h at room temperature). The samples were then rinsed once with blocking buffer and twice with PBS (15 min each, at room temperature) before they were mounted on slides using FluorSave® antifade mounting medium (Calbiochem). The samples were oriented as such the stereocilia of hair cells pointing upward. Specific labeling was initially examined with a field fluorescence microscope and further detailed images were obtained using a confocal laser scanning microscope (Nikon). The types and conditions of the antibodies used in this study were as following: rabbit anti-ANO1

(ABcam, AB53212, 1:500), polyclonal anti-Bestrophin-1 (Young In Frontier, 1:200), monoclonal anti-NKCC1 (Hybridoma bank, T4, 1:1000), monoclonal anti-calretinin (Chemicon, AB5054, 1:1000), Alexa-488 goat anti-rabbit (Molecular Probes, 1:1000), Alexa-488 donkey anti-rabbit (Molecular Probes, 1:1000), Alexa-555 goat anti-mouse (Molecular Probes, 1:1000), Alexa-555 donkey anti-mouse (Molecular Probes, 1:1000), Alexa-549 goat anti-rabbit (Molecular Probes, 1:1000).

For spontaneous volume change recording, freshly isolated cochleae were placed under microscope (Olympus BXWI) equipped with 40X water immersion objective and DIC optics. Using Image Workbench 6 software (INDEC Biosystem), images were obtained at 1 frame per sec. After making adjusted images by subtracting frames captured at times t_n and t_n+_{5sec}, the regions exhibiting optical changes were quantified using ImageJ software (NIH).

RESULTS

Under differential interference contrast (DIC) optics the changes in GER supporting cell shape and light transmittance intensity around them could be easily imaged. Similar to findings in previous study [9], spontaneous optical changes were recorded in the GER of P9-11 mouse cochlea during control period. A chloride channel blocker, 100 μM NPPB, inhibited the optical changes (baseline: 8.5±1.7, NPPB: 0.5±0.3 time/20 min) (Fig. 1). The result is consistent with the previous finding that Cl^- channel is involved in the spontaneous activities of GER supporting cell.

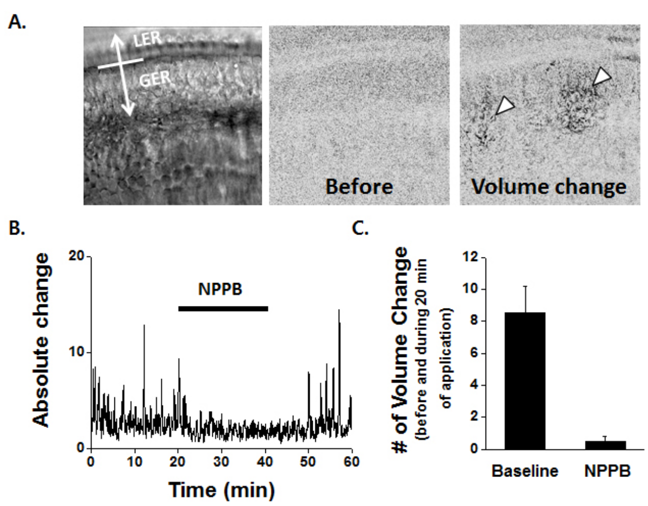

Fig. 1. Spontaneous activity in GER supporting cells is inhibited by anion channel blocker, NPPB. (A) Spontaneous volume change was observed in cochlea. Areas exhibiting cell volume change were indicated with white arrows in the images from before and during volume change. (B) Plot of quantification of spontaneous volume change in greater epithelial ridge (GER). During NPPB application for 20 min the change was inhibited and recovered after washout of the drug. (C) Summary bar graph shows inhibitory effect of NPPB (100 μM) on the spontaneous volume change in cochlea (n=4 each).

Based on this pharmacological evidence, we began our investigation on 2 known Ca^{2+}-activated chloride channels and a chloride transporter that has been previously reported to mediate Cl^- flux in the cochlea. Ano1 has been found in inner sulcus of embryonic inner ear [16], stria vascularis and efferent nerve terminals contacting OHCs in adult cochlea [17]. Na^+, K^+, Cl^- co-transporter 1 (NKCC1) has also been found in stria vascularis of adult cochlea. NKCC1 knockout mice are profoundly deaf, with scala media collapsed [18]. However, if these channels are expressed in postnatal, developing cochlea has not been investigated in detail.

The expression of Ano1, Bestrophin-1 and NKCC1 in the cochlea was tested by immunofluorescence labeling. Cochlear preparations from P9-10 mice were first tested because spontaneous supporting cell volume change had been shown to reach its peak around P9 [9]. High level of Ano1-immunoreactivity (IR) was found in the region near the row of IHCs (Fig. 1A, C, D, F). On the other hand, Bestrophin-1-IR and NKCC1-IR were only found in interdental cell area (Fig. 2B, E). Therefore, expression of Ano1 was further investigated. To confirm that Ano1 is expressed in the GER region, double-immunolabeling with calretinin was performed. Calretinin is a Ca^{2+} binding protein expressed in the cytosol of hair cells and auditory afferent nerve fibers [19]. Three rows of OHCs, one row of IHCs, and auditory neurons and their thin peripheral processes could be visualized through calretinin-IR (Fig. 2H, I). Strong Ano1-IR was observed in the region near the row of calretinin-positive IHCs and its modiolar vicinity (Fig. 2G, I), further supporting the Ano1 expression in GER. Apart from the cells in GER, stereocilia of inner or outer hair cells also exhibited occasional fluorescence labeling. The labeling pattern of stereocilia, however, was not consistent even among cochlear preparations prepared on the same day under identical condition. Thus, we could not exclude the possibility of non-specific reaction of the antibodies to the stereocilia. Except occasional stereociliary staining, no specific immunolabeling beyond auto-fluorescence was found in LER.

In the GER of developing cochlea, inner hair cells, auditory nerve fibers as well as various types of supporting cells are in close contact with one another. However, observable volume changes had been found only in supporting cells [8]. Thus, whether the Ano1-IR is localized only in the supporting cells was tested by examining high magnification confocal images. Ano1-expressing cells appeared to surround a row of unstained cells (Fig. 3A, B). Judging from their shape and location, these unstained cells

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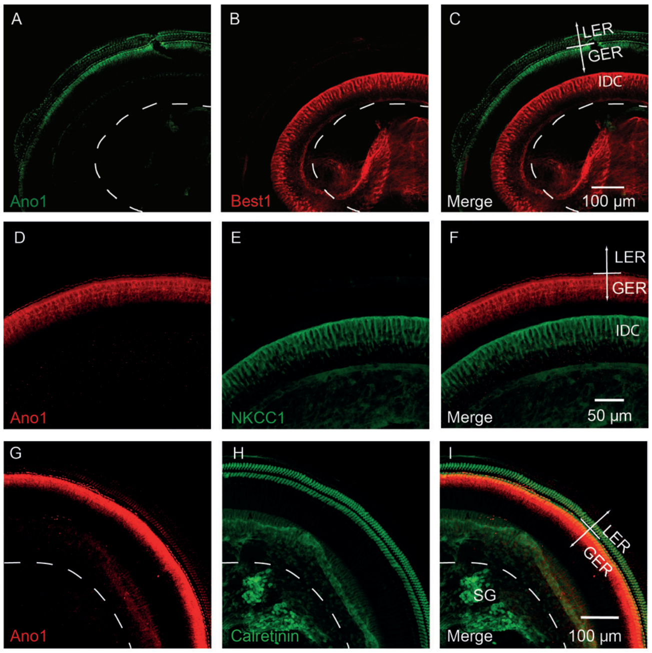

Fig. 2. Ano1 is expressed in the GER of the developing mouse cochlea. (A~C) Ano1 (Green) and Bestrophin-1 double immunofluorescence labeling of P10 cochlear whole-mount preparation. (D~F) Ano1 (red) and NKCC1 (green) double immunofluorescence labeling of P9 cochlear whole-mount preparation. Ano1 immunoreactivity was found in greater epithelial ridge (GER) whereas Bestrophin-1 and NKCC1 immunoreativities were limited to the interdental cell area (IDC). (G~I) Ano1 (red) and calretinin (Green) double immunofluorescence labeling of P9 cochlear whole-mount preparation. One row of inner hair cells, three rows of outer hair cells, and spiral ganglion neurons (SG) and their peripheral processes are visualized by calretinin-immunoreactivity. Ano1 Immunoreactivity is found only near inner hair cells but not near the outer hair cells. A, B, D, E, G and H are maximal projection renderings of confocal z stack slices. C, F and I are merged views of A and B, D and E, and G and H, respectively.

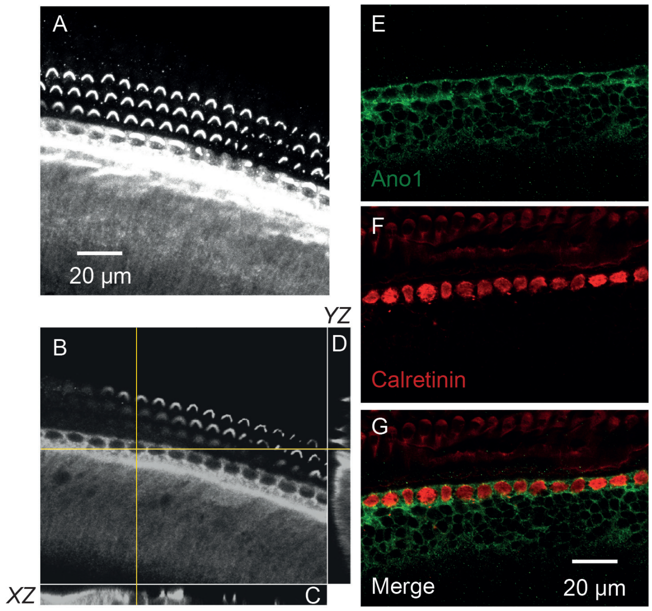

Fig. 3. Ano1 is expressed in GER supporting cells. (A~D) High magnification confocal images of Ano1 immunofluorescence labeling acquired from P9 cochlear whole-mount preparation. (A) Maximal projection rendering of confocal Z stack slices. (B) Single confocal slice from middle of confocal z stack in A. (C, D) The XZ (C) and YZ (D) projection images at the location marked in B with yellow vertically-crossing lines. The stereociliary bundles of the hair cells are not consistently labeled thus, stereociliary stainings shown in A and B are thought to be non-specific reaction. (E, F) Single confocal slice images of Ano1 and calretinin double immunolabeling from another P10 cochlear whole-mount preparation. (G) Merged view of E and F. There was no overlap between Ano1 and calretinin labelings.

were thought to be IHCs. To further confirm that Ano1-IR is limited in support cells, confocal slice images following double-immunolabeling with calretinin were examined. Ano1-IR was observed in cells circumscribing the calretinin-expressing IHCs as well as in their modiolar neighbors (Fig. 3E, F). No significant overlap was observed between calretinin-IR and Ano1-IR, indicating Ano1 is expressed in supporting cells surrounding IHCs but neither in IHCs nor auditory nerve fibers.

Supporting cell volume changes in the cochlea are prominent but transient phenomena, observed during early postnatal weeks [9]. Immediately after the birth, supporting cell volume changes occurred at low frequency in cells closely contacting IHCs. The frequency and area of this phenomenon gradually increased over the first postnatal week, reached its peak around P9. Then, it rapidly diminished after hearing onset (~P11). After the second postnatal week almost no supporting cell volume change was recorded. Thus, the time course of Ano1 expression in the cochlea was next investigated. The cochleae from P2-3, 5-6, 9-10, 15-17 mice were compared (Fig. 4). In cochleae from P2-3 mice Ano1-IR was found in cells relatively near the IHC row (Fig. 4A). As the cochleae mature (P5-6, P9-10) the area expressing Ano1 appeared to be broadened toward the modiolar direction (Fig. 4B, C). In contrast, the cochleae from P15-17 mice (post-hearing onset) exhibited no Ano1-IR in the GER supporting cells (Fig. 4 D). These data indicate that the Ano1 expression in GER, like the supporting cell volume changes in developing cochlea, is transient phenomenon, occurring until immediately after the hearing onset.

DISCUSSION

Ano1 and its various physiological roles

Ano1 was first identified as a transmembrane protein conferring Ca^{2+}-activated Cl^- channel activity by 3 independent research groups in 2008 [20-22]. Hydropathy analysis prediction suggested that Ano1 has 8 transmembrane domains, a p-loop between

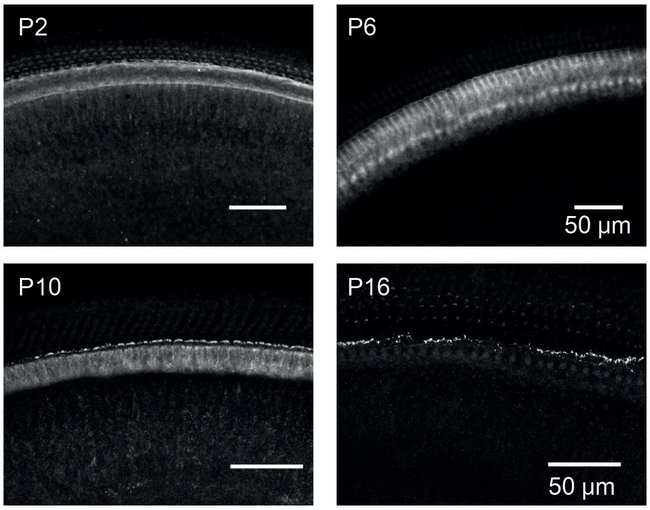

Fig. 4. Developmental changes in Ano1 expression. Ano1 immunoreactivity in P2, P6, P10, and P16 cochlear whole-mount preparations was compared. Ano1 expression was observed in P2, P6, and P10 cochlear preparations but mostly disappeared in P16 cochlea. All images are maximal projection renderings of confocal z stack slices.

TM5 and 6, cytosolic N and C-termini. Its activity modulation by cytosolic Ca^{2+} concentration, calmodulin, and IP_3 has been documented [21, 23].

Prior to its full identification, Ano1 had been known by different names, such as DOG-1 (discovered on gastrointestinal stromal tumors protein 1), ORAOV2 (oral cancer overexpressed 2), and TAOS2 (tumor-amplified and overexpressed sequence2) due to its high expression in several cancer cells [24, 25]. More recent study elucidated that Ano1 is also found in breast cancer cells, where it promotes cancer progression by stimulating cell proliferation signaling pathway involving EGFR and CAMK [26].

However, Ano1 participates in a wide variety of normal cellular functions as well. In the interstitial cell of Cajal (ICC) of the gastrointestinal tract and the oviduct Ano1 acts as a pacemaker for spontaneous motility [27, 28]. In the epithelial cells of trachea, pancreas, salivary gland and biliary tract, it plays significant role in fluid secretion [29, 30] and cell volume regulation upon osmotic stress [31]. Of note, the Ano1 function in fluid secretion/cell volume regulation appeared to be coupled to ATP release and subsequent purinergic receptor activation [21, 29, 32].

Involvement of Ano1 is also implicated in sensory functions. Its presence has been reported in the presynaptic terminals of retinal neurons [33], vomeronasal sensory epithelium [34, 35], and dorsal root ganglion neurons [36]. In adult mouse cochlea Ano1 is highly expressed in stria vascularis [17], and thus, the channel is thought to participate in maintaining Cl^- homeostasis of the endolymph. Indeed, gene mutations causing loss of Cl^- homeostasis in endolymph result in hearing deficit either because the endolymph is not formed properly [18] or the endocochlear potential is lost despite the normal K^+ concentration in the endolymph and intact cochlear structures [37]. Ano1 expression has been also found in the GER of embryonic cochlea [16], suggesting its potential role in the development of auditory epithelium itself. Although Ano1 KO animals had been generated it is still unclear whether Ano1 is necessary for the function and/or the structural development of auditory organs. Auditory organ functions of Ano1 KO animals had not been reported because the Ano1 KO animals die too early (for auditory function test) after birth due to tracheal development defect [38].

Spontaneous, periodic activity of GER supporting cells in neonatal cochlea

GER supporting cells in developing cochlea exhibits peculiar spontaneous electrical activity. This spontaneous activity appears to be present from birth until shortly after hearing onset and involves a series of ATP-mediated signaling events; ATP release from 1-2 supporting cells through connexin hemichannels, activation of P2X, P2Y receptors in autocrine and paracrine fashion, increase in cytosolic Ca^{2+} concentration directly by influx through P2X receptor and indirectly via IP_3 generation and subsequent Ca^{2+} release from intracellular stores, spreading of Ca^{2+} and second messengers to surrounding supporting cells through gap junction coupling, more ATP release and further propagation until ATP degradation by extracellular nucleotidases [7-9].

Surprisingly, very similar ATP-mediated signaling events occur in the LER supporting cells as well although not spontaneous [12]. Then, what makes such difference in spontaneity? One possible explanation is that supporting cells in GER and LER express different subtypes of connexin hemichannels; i.e. GER with high opening probability, whereas LER with low opening probability. However, evidence presented so far report otherwise. Supporting cells in GER and LER express the same subtypes of connexins, namely connexin 26 and connexin 30 [14]. Disruption of either connexin 26 or connexin 30 resulted in diminished spontaneous ATP-mediated Ca^{2+} signaling in GER as well as ATP induced Ca^{2+} signaling in LER [12, 39]. Likewise, loss of spontaneous ATP signaling in mature GER supporting cells is not due to lack of connexin hemichannels or purinergic receptors. ATP release from connexin hemichannel can occur in adult cochlea supporting cells [40]. The GER supporting cells remain responsive to exogenous ATP after hearing onset [9]. Taken together, all these findings support a presence of pacemaker molecule that is transiently expressed in the GER supporting cells and triggers hemichannel opening. Here, we have shown that Ano1, the pacemaker of

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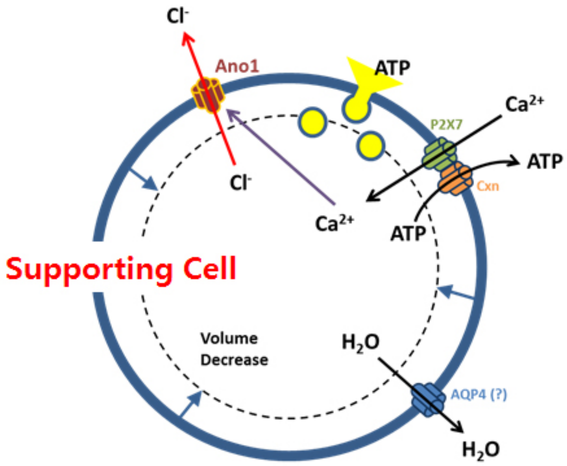

Fig. 5. Schematic diagram of spontaneous volume regulation in developmental supporting cell.

spontaneous electrical activity in GI tract and oviduct [27, 28], is also expressed in GER supporting cell in neonatal cochlea before hearing onset. The cellular localization and time course of Ano1 expression suggests that Ano1 might acts as a pacemaker of spontaneous activity in developing cochlea.

GER supporting cell volume change

Why does the spontaneous activity of GER supporting cell accompany such prominent volume change? So far, the key link between the GER supporting cell volume change and the spontaneous electrical activity is Cl^- flux. Both phenomena were inhibited by nonspecific Cl^- channel blockers such as NPPB (Fig. 1) and DIDS [8]. The cell volume change in GER supporting cells could be a simple byproduct of water movement following Cl^- flux (Fig. 5). At least cell volume change does not appear to be absolute necessity for ATP release and subsequent modulation of hair cell functions. In LER, without observable supporting cell volume change, enough amount of ATP can be released to modify the OHC function [40, 41]. However, more attractive hypothesis is also possible. Tritsch et al. [8] proposed that fluid secretion along the Cl^- flux might contribute to 1) maturation of ionic composition of endolymph and perilymph solutions during prehearing developmental stage, 2) detachment of the low-lying tectorial membrane to its final position, or 3) mimicking sound wave-generated basila membrane movement and thus, acclimatizing the hair cells to the movement of the surrounding structures before the real sound wave can reach them (Before hearing onset, the sound wave cannot be transmitted to the cochlea hair cells due to fluid-filled middle ear). Alternatively, the supporting cell volume change might be a device for rapidly transporting signaling molecules such as growth factors. It is well documented that initial two postnatal weeks in rodents are critical period of the cochlea development. Complex actions of many growth factors and hormones are required for the maturation of sensory hair cells and the proper synapse formations between hair cells and auditory afferent and efferent neurons [6, 42]. Rapid forming and closing of extracellular space around inner hair cells may facilitate transportation of signaling molecules to the area otherwise so heavily protected by layers of supporting cells. It is not clear whether any cytoskeleton shifting motor protein is involved in the supporting cell volume change.

ACKNOWLEDGEMENTS

This study was supported by Basic Science Research Program through the National Research Foundation of Korea funded (2012009971) to Eunyoung Yi. This work was also supported by the World Class Institute (WCI) Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (MSIP) (NRF Grant Number: WCI 2009-003) and National Agenda Project (NAP) of the Korea Research Council of Fundamental Science & Technology (NAP-09-04) for Dr. C Justin Lee.

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