Published in final edited form as:

Brain Res. 2006 June 20; 1095(1): 51–58. doi:10.1016/j.brainres.2006.04.017.

Prestin is expressed on the whole outer hair cell basolateral surface

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Abstract

Prestin has been identified as a motor protein responsible for outer hair cell (OHC) electromotility. Previous experiments revealed that OHC electromotility and its associated nonlinear capacitance resided in the OHC lateral wall and was not detected at the apical cuticular plate and basal region. In this experiment, the distribution of prestin in adult mouse, rat, and guinea pig OHCs was re-examined by use of immunofluorescent staining and confocal microscopy. We found that prestin labeling was located at the whole OHC basolateral wall, including the basal plasma membrane. However, staining at the basal membrane was weak. As compared with the intensity at the lateral wall, the intensities of prestin labeling at the membrane at the nuclear level and basal pole were 80.5% and 61.1%, respectively. Prestin labeling was not found at the cuticular plate and stereocilia. The prestin labeling was also absent in the cytoplasm and nuclei. The OHC lateral wall above the nuclear level is composed of the plasma membrane, cortical lattice, and subsurface cisternae. By costaining with di-8-ANEPPS, prestin labeling was found at the outer layer of the OHC lateral wall, which was further evidenced by use of a hypotonic challenge to separate the plasma membrane from the underlying subsurface cisternae. The data revealed that prestin is expressed at the whole OHC basolateral membrane. Prestin in the basal plasma membrane may provide a reservoir on the OHC surface for prestin-recycling and may also facilitate performing its hypothesized transporter function.

Keywords

Electromotility; Plasma membrane; Cochlea; Active mechanics; di-8-ANEPPS

1. Introduction

Mammalian hearing function relies upon active motility of outer hair cells (OHCs) to boost the basilar membrane vibration (Brownell et al., 1985; Dallos, 1992). The OHC has a cylindrical shape; its apical pole has the cuticular plate which stereocilia are located on, and its basal pole contains a nucleus and has synapses connected with auditory nerves. The OHC lateral wall has a unique trilaminar organization above the nuclear level and is composed of plasma membrane (PM), cortical lattice (CL), and subsurface cisternae (SSC) (Flock et al., 1986; Forge, 1991; Forge et al., 1993; Holley and Ashmore, 1990; Holley et al., 1992; Oghalai et al., 1998). The PM is the outermost layer of the lateral wall. The CL is located beneath the PM and is an orthotropically organized cytoskeletal structure. The SSC is the innermost layer and is composed of endoplasmic membranous laminates (Saito, 1983). The CL and SSC line the lateral cytoplasmic surface of the plasma membrane and terminate above the nuclear level.

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Previous experiments demonstrated that OHC electromotility resided in its lateral wall (Dallos et al., 1991; Kalinec et al., 1992; Hallworth et al., 1993; Gale and Ashmore, 1997a). Patch clamp recording also showed that the motility-associated nonlinear capacitance was small and undetectable at the subnuclear membrane (Huang and Santos-Sacchi, 1993; Gale and Ashmore, 1997b). It was assumed that motor protein had no distribution in the plasma membrane below the nuclear level (Hallworth et al., 1993; Huang and Santos-Sacchi, 1993).

Recently, prestin has been identified as a motor protein responsible for OHC electromotility (Zheng et al., 2000; Liberman et al., 2002). Immunofluorescent staining of OHCs for prestin in the whole-mount organ of Corti showed a ring-labeling pattern at the confocal scanning section orthogonal to the OHC longitudinal axis, indicating that prestin is expressed on the OHC surface (Belyantseva et al., 2000; Zheng et al., 2001, 2003; Adler et al., 2003). However, the precise distribution of prestin on the OHC surface lacks detailed description. In this experiment, we re-examined prestin expression in OHCs by use of immunofluorescent staining and confocal microscopy with whole epithelium mounting preparation and single dissociated cell preparation. We found that prestin labeling was visible on the whole OHC basolateral wall. In the lateral wall, strong prestin labeling was located in the plasma membrane.

2. Results

Outer hair cells (OHCs) have three rows in the cochlear sensory epithelium. Figs. 1A–D show immunofluorescent staining of OHCs in situ for anti-prestin in whole-mount preparation. Prestin labeling showed a characteristic ring pattern and appeared 3 rows in the OHC area. However, there was no labeling found in the inner hair cell and supporting cell area (Figs. 1A–D). This negative staining served as a good internal control for specificity of staining to prestin. Figs. 1A–C are the serial confocal images scanned at different Z depths at the OHC nuclear level. The cell nuclei were revealed by co-staining with DAPI. As the scanning section was going down, the nuclei of the 1st, 2nd, and 3rd row OHCs consequently appeared and then disappeared (Figs. 1A–C). Prestin labeling was clearly visible at the nuclear level and encircled the nuclei. Fig. 1D is the optical transversal section of confocal image. Prestin labeling could be seen along the OHC lateral wall from top to bottom including at the nuclear level (indicated by arrows in Fig. 1D). An arrow in an inset in Fig. 1D indicates prestin labeling at the basal pole.

In order to reveal prestin expression on the OHC surface clearly, we examined immunofluorescent staining of dissociated OHCs for anti-prestin. Figs. 1E and F show prestin labeling in a dissociated OHC. Prestin labeling was visible at the whole basolateral membrane, including the subnuclear membrane at the nuclear level and basal pole. However, there was no prestin labeling at the apical cuticular plate (Fig. 1E) and a connected supporting cell (indicated by an arrow in Fig. 1F), which is known as having no prestin expression. Prestin distribution at the subnuclear membrane at the nuclear level and basal pole was found in all examined OHCs ($n > 100$, length range: 18–80 μm).

Prestin labeling at the subnuclear membrane was also visible in mouse and rat OHCs (Fig. 2). Figs. 2A, C, and E show that OHC basolateral membrane had strong prestin labeling but the stereocilia had no labeling (indicated by an arrow in Fig. 2B). A broken Deiters cell's stalk in Figs. 2C and D also had no prestin labeling. Prestin labeling in the OHC subnuclear region was also found in mouse and rat epithelia in whole-mount preparation (data not shown).

Immunofluorescent staining of OHCs for antibody to prestin N-terminus shows the same labeling pattern as the staining for anti-prestin C-terminus (Fig. 3). Prestin labeling was observed on the surface of whole OHC basolateral wall, but absent in the cytoplasm and nuclear membrane (Figs. 3A and C). In order to verify whether prestin antibody penetrated through

the plasma membrane into the cytoplasm, we used di-8-ANEPPS to co-stain the cells. The dye di-8-ANEPPS is a plasma membrane impermeable dye labeling the phospholipid bilayer. After fixation and treatment with Triton X-100 in immunofluorescent staining, di-8-ANEPPS could pass through the plasma membrane and labeled the cytoplasmic membranous organelles and the nuclear membrane (Fig. 3B). However, there was no prestin staining in cytoplasm and nucleus (Fig. 3A). An arrow in Fig. 3C indicated that prestin labeling was located at the separated plasma membrane instead of the nuclear membrane at the basal pole.

Prestin labeling at the subnuclear membrane appeared weak (Figs. 1–3). We quantitatively analyzed distribution of prestin labeling on the OHC surface (Fig. 4). The intensities of prestin labeling at the lateral wall, the membrane at the nuclear level, and the basal membrane (see an inset in Fig. 4A) were measured and normalized to the staining intensity of the lateral wall in each cell. In the dissociated cells, the normalized intensities at the nuclear level and basal membrane were $80.49 \pm 3.72\%$ and $61.11 \pm 5.55\%$, respectively (Fig. 4A), and were significantly less than that at the lateral wall ($P < 0.001$, paired t test). In the whole-mount preparation, the normalized intensity of prestin labeling at the subnuclear membrane (the membrane at the nuclear level and basal pole) was $61.00 \pm 6.12\%$ (Fig. 4B), which was less than $70.80 \pm 4.13\%$ measured in the dissociated cells but there was no statistically significant difference between them ($P = 0.24$, t test).

The OHC lateral wall is composed of the PM, CL, and SCC above the cell nucleus. The di-8-ANEPPS dye labeled the PM as well as other cell membranous structures, including SCC, endoplasmic reticulum, and stereocilia, after fixation and treated with Triton X-100 (Fig. 5B). Prestin labeling overlapped di-8-ANEPPS staining in the basolateral wall, but not in the endoplasmic reticulum and stereocilia (Fig. 5C). At high magnification (inset in Fig. 5C), prestin labeling was present at the outermost, most likely PM layer in the OHC lateral wall, but was absent at the inner layer, which was indicated by the empty white arrows.

In order to further examine the localization of prestin in the OHC lateral wall, we applied a hypotonic extracellular solution to induce OHC swollen, attempting to separate the PM from the underlying SSC and CL (Oghalai et al., 1998). An arrow head in Figs. 6A–C indicated that hypotonic challenge induced cell swollen and formed a bubble on the OHC lateral wall; strong prestin labeling was visible at the bubble edge. However, there was no prestin labeling in the underlying SSC. This is consistent with previous reports that the OHC motor protein is located within the plasma membrane in the OHC lateral wall (Kalinec et al., 1992; Huang and Santos-Sacchi, 1994; Santos-Sacchi and Zhao, 2003). Figs. 6D–F also show that as the lateral wall was ruptured by the hypotonic challenge, prestin labeling was absent at the ruptured mouth (indicated by arrows).

3. Discussions

In this experiment, we found that prestin is expressed on the whole OHC basolateral surface. Prestin labeling was clearly visible at the subnuclear region in whole-epithelium mounting preparation as well as in single dissociated cell preparation (Figs. 1–6). Immunofluorescent staining showed the same labeling pattern for anti-prestin C-terminus and N-terminus antibodies in three different species (guinea pig, rat, and mouse), and had no labeling in the inner hair cells and supporting cells (Figs. 1–3), indicating a high specificity of staining to prestin.

3.1. Expression of prestin in the OHC subnuclear region

The distribution of prestin in the subnuclear membrane in OHCs is inconsistent with previous conception that the distribution of prestin is restricted at the supernuclear membrane of the OHC lateral wall. It has been reported that prestin labeling was not visible at the OHC nuclear

level in the cochlear sensory epithelia in whole-mount preparation (Belyantseva et al., 2000). However, cell nuclei were not demonstrated in that experiment. So, the OHC nuclear region could not be precisely determined. In this experiment, cell nuclei were demonstrated by co-staining with DAPI. Prestin labeling was clearly visible at the nuclear level and encircled the OHC nuclei in whole-mount preparation (Figs. 1A–C). Subnuclear labeling of prestin was further demonstrated in the optical transversal cross-section of confocal image in whole-mount preparation (Fig. 1D). Prestin labeling at the OHC subnuclear plasma membrane could also be seen in the cross-section of the rat cochlea in previously published data (see Fig. 6 in Weber et al., 2003; Figs. 6A and 7A in Ruttiger et al., 2004). Thus, prestin *in situ* has subnuclear distribution.

As compared with the labeling intensity in the OHC lateral wall, however, prestin distribution in the subnuclear region was weak (Figs. 1–3). Quantitative analysis showed that in whole-mount preparation prestin staining in the subnuclear membrane only had 61% of intensity of the lateral wall (Fig. 4B). *In situ*, the basal ends of OHCs sit on the cups of Deiters cells and are embedded by synapses formed with auditory nerve endings. This could prevent antibody accessing and binding in the OHC basal portion. In the present experiments, as the OHC basal pole had wreckages of cell membranes or nerve endings covered or attached, prestin labeling appeared weak or absent (Figs. 2C and D, 3C, 5C, and 6F). On the other hand, the OHC basal pole also possesses a high density of ionic channel distribution (Santos-Sacchi et al., 1997). This could exclude prestin expression as well, resulting in weakly staining for prestin in the OHC basal membrane.

Prestin might laterally diffuse down to the nucleus level after dissection. The lateral diffusion in the OHC lateral wall has been reported (Oghalai et al., 2000; Santos-Sacchi and Zhao, 2003). For some unknown mechanisms, the prestin proteins may be restricted *in vivo* and could not diffuse down to the nucleus level. Dissection might remove the restriction allowing prestin proteins diffusing down to the basal pole. However, prestin expression could be seen in the OHC basal region *in situ* in whole-mount preparation (Figs. 1A–D). The staining intensity in the subnuclear region in the whole-mount preparation was slightly less than in dissociated cell preparation (Figs. 1–3). Quantitative analysis showed that there was no significant difference (Fig. 4B), even antibody may have some restrictions to access the OHC basal pole *in situ*. Thus, dissociating process did not significantly contribute to prestin expression in the subnuclear region.

In the dissociated OHCs, prestin labeling was clearly demonstrated at the subnuclear membrane (Figs. 1E and F and Figs. 2–6). However, patch-clamp recording could not find or detect electromotility or nonlinear capacitance at the basal pole of in the dissociated OHCs (Dallos et al., 1991; Hallworth et al., 1993; Huang and Santos-Sacchi, 1993; Gale and Ashmore, 1997b). Several reasons could result in this discrepancy. First, because the prestin expression in the basal pole is weak (Fig. 4), the nonlinear capacitance could be too small to be detected in the patch recording. Second, since the OHC subnuclear region lacks the CL and SSC structures, the membrane movement driven by prestin may become small and undetectable. It has been reported that in prestin transfect cells cell membrane movement is very small and almost undetectable (Zheng et al., 2000). Finally, prestin proteins in the basal pole may have no ‘function’ for electromotility. Recently, it has been found that prestin is also expressed in the vestibular hair cells of rodent saccule, utricle, and crista ampullaris, but has no electromotility and nonlinear capacitance (Adler et al., 2003). So, nonmotility prestin may also exist in the cochlear OHC basal pole.

3.2. Possible nonmotility functions of prestin in the OHC subnuclear region

Several nonmotility functions can be hypothesized for prestin localized at the subnuclear membrane. First, the prestin proteins in the subnuclear membrane may provide a reservoir in

the OHC basolateral wall. Recently, we reported that prestin can be regulated by administration of salicylate and may be in continuously recycling in life span (Yu et al., 2005). Prestin in the basal region may provide a reservoir for prestin recycling.

Second, prestin in the basal membrane may facilitate performing transporter function. Recently, it has been hypothesized that prestin may also function as a transporter besides electromotility (Chambard et al., 2003). The *prestin* gene belongs to a sulfate/anion transporter family and has high homology to the members of the SLC26 family of anion transport proteins (Zheng et al., 2000, 2001). The OHC basal pole possesses a high density of ionic channels (Santos-Sacchi et al., 1997) and synapses formed by the auditory nerve endings. Prestin located in the basal membrane can perform transporter function more efficiently.

Finally, it has been hypothesized that prestin may play an important role in neurotransmitter vesicle release (Brownell et al., 2001). Prestin expression in the basal membrane provides a histological evidence for this hypothesis, enhancing neurotransmitter release by amplification of flexoelectrical forces.

4. Experimental procedures

4.1. Animal and outer hair cell preparation

A total of 21 albino guinea pigs (200–450 g), 3 Sprague–Dawley rats (6–8 weeks), and 10 CBA mice (6–10 weeks) were used in this experiment. After injection of overdose of Pentobarbital, animals were decapitated and the temporal bones were removed. The otic capsule was dissected in a standard extracellular solution (130 NaCl, 5.37 KCl, 1.47 MgCl₂, 2 CaCl₂, 25 Dextrose, and 10 HEPES in mM; 300 mOsm and pH 7.2) to reveal the organ of Corti. After the tectorial membrane and stria vascularis were removed, the sensory epithelium (organ of Corti) was picked away with a sharpened needle. The isolated epithelia were transferred to a dish for staining. For the dissociated cell preparation, the isolated sensory epithelia were further dissociated with the enzyme trypsin (1 mg/ml) for 5–10 min. The dissociated cells were then transferred to a dish for staining. All experimental procedures were conducted in accordance with the policies of University of Kentucky's Animal Care and Use Committee.

4.2. Prestin antibodies

Two polyclonal rabbit anti-mouse-prestin C-terminal fragment (Ab#792) and N-terminal fragment (Ab#802) antibodies were used in this experiment (kind gift of Dr. Zheng, Northwestern University). The specificity of these antibodies has been fully characterized and verified in previous studies, including in prestin knockout mice (Zheng et al., 2001, 2005; Matsuda et al., 2004). In later experiments, we also used other two commercial anti-prestin C-terminal and N-terminal antibodies (cat#: ss-22694 and ss-22692, respectively, Santa Cruz Biotechnology Inc., CA). There was no difference found for all used anti-prestin antibodies in staining.

4.3. Immunofluorescent staining

The dissected cochlear sensory epithelia or dissociated cochlear cells were fixed with 4% paraformaldehyde for 30 min. After washing with 0.1 M PBS for 3 times, the epithelia or cells were incubated in a blocking solution (10% goat serum and 1% BSA in the PBS) with 0.1% Triton X-100 for 20 min. Then, the epithelia or cells were incubated with an anti-prestin antibody (1:1000–1500) in the blocking solution at 4°C overnight. In control experiments, the anti-prestin antibody was omitted. After washing with PBS for 3 times, the cells were reacted to an Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Cat. A11034, Molecular Probes) in the blocking solution at room temperature for 1 h. For co-staining with the dye di-8-ANEPPS to demonstrate the plasma membrane and cytoplasmic membranous organelles, the epithelia

or cells were further incubated in 30 μ M di-8-ANEPPS (D-3167, Molecular Probes Inc., Eugene, OR) for 2–3 min after the secondary antibody incubation. After completely washing dye out with 0.1 M PBS, the staining was observed under a confocal microscope.

4.4. Cellular nucleus staining

The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, D1306; Molecular Probes). The stock solution of DAPI (5 mg/ml) was made with deionized water. Following the reaction to the 2nd antibodies, pieces of epithelia or dissociated cells were incubated with a 1:50 dilution of DAPI stock solution at room temperature (23 °C) for 15–30 min, and washed with PBS for 3 times.

4.5. Confocal laser-scanning microscopy

The stained epithelia or cells were observed under a Leica confocal microscope (Leica TCS SP2) equipped with a 100 $\times$ (NA 1.4) apochromatic oil objective. The argon (488 nm) laser with 496–530 nm and 630–710 nm emission filters was used for the visualization of Alexa Fluor 488 and di-8-ANEPPS, respectively. The DAPI staining was watched by use of a UV 2-photon with a 380–484 nm emission filter.

4.6. Quantitative analysis of staining

The immunofluorescent staining of prestin in OHCs was quantitatively analyzed by NIH image software (Bethesda, MD). The staining intensities at the OHC lateral wall above the nuclear level, at the nuclear level, and at the basal pole membrane were separately measured. The intensities were then normalized to the staining intensity at the lateral wall and averaged. The data were presented as mean $\pm$ SE.

4.7. Hypotonic challenge

Cell hypotonic challenge was achieved by application of a hypotonic extracellular solution. Dissociated cells were incubated in a 275 mOsm hypotonic extracellular solution for 5 min prior to fixation. The solution osmolarity was adjusted by Dextrose and measured by a micro-computer controlled osmometer (Model 3300, Advanced Instruments Inc. Norwood, MA).

Acknowledgements

We are grateful to Dr. Jing Zheng at Northwestern University for providing anti-prestin antibodies. We thank P.G. Wilson and Ni Ji for technical support. This work was supported by NIDCD DC 05989 and American Tinnitus Associate Research Foundation.

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Fig. 1.

Immunofluorescent staining of guinea pig outer hair cells (OHCs) for prestin in in situ whole-mount preparation and in dissociated cell preparation. (A–C) Serial confocal scanning sections of the auditory sensory epithelium in whole-mount preparation. The sections were taken at the OHC nuclear level down to the basal bottom; three rows of OHCs consequently disappeared. Green and blue colors represent prestin labeling and DAPI staining for cell nuclei, respectively. Prestin staining shows a ring-labeling pattern only in the OHC area. (D) An optical transversal section of confocal image of immunofluorescent staining of the epithelium for prestin. Arrows indicate the prestin labeling at the subnuclear membrane. Inset: An arrow indicates strong staining of prestin at the basal pole membrane at the differing focus level. (E and F) Prestin staining of a dissociated OHC. Prestin labeling is clearly visible on the whole basolateral surface. An arrow indicates a collapsed Deiters cell that has no fluorescent staining. Scale bars: 20 μm .

Fig. 2. Immunofluorescent staining of prestin in mouse and rat OHCs. OHCs were dissociated from the mouse or rat cochlea and were staining for anti-prestin C-terminus. Prestin labeling is visible at the subnuclear membrane but not at stereocilia and the cuticular plate. Note that the OHC in panels A and B was slightly shrunk. An arrow in panel E indicates prestin labeling in mouse OHC basal membrane. Scale bar: 10 μ m.

Fig. 3.

Immunofluorescent staining of an OHC for anti-prestin N-terminus. Panel A is a confocal image of immunofluorescent staining for prestin. Strong staining is located on the surface of the cell basolateral wall; there is no prestin labeling in the cytoplasm and nucleus. Panel B is a fluorescent image for co-staining with di-8-ANEPPS. Positive staining is visible at the plasma membrane and membranous organelles in the cytoplasm. Panel C is a merged image. A white arrow points to that the prestin-labeled plasma membrane is divorced from the nuclear membrane. Panel D is the Nomarski image. Scale bar: 15 μm.

Fig. 4. Quantitative analysis of the membrane distribution of prestin on the OHC surface. (A) Distribution of prestin expression on the dissociated OHC surface. The intensities of prestin labeling at the membrane at the lateral wall, the nuclear level, and the basal pole (see inset) were measured and normalized to the labeling intensity of the lateral wall in each cell. Stars indicate that there is a statistically significant difference ($P < 0.001$, paired t test). (B) Comparison of prestin labeling at the OHC subnuclear membrane in dissociated cell preparation and in whole-mount preparation. The subnuclear intensity was averaged from the labeling intensities at the nuclear level and the basal pole. There was no significant difference in expression of prestin at the subnuclear membrane between two preparations ($P = 0.24$, t test).

Fig. 5.

Expression of prestin in the OHC basolateral wall. An OHC was co-stained for prestin and di-8-ANEPPS. Panels A and B are fluorescent images for prestin labeling and dye di-8-ANEPPS staining, respectively. Panel C is a merged image. Prestin labeling and di-8-ANEPPS staining appear overlapped on the surface of the OHC basolateral wall. The stereocilia and cuticular plate have di-8-ANEPPS staining but no prestin labeling (indicated by an arrow head and arrows, respectively). Inset: A high magnification image. Empty arrowheads indicate the cytoplasmic surface of the lateral wall that has no prestin labeling. (D) The Nomarski image in the same field. Scale bar: 15 μ m; inset: 1 μ m.

Fig. 6.

Co-staining of OHCs for prestin and di-8-ANEPPS with a hypotonic challenge. The isolated OHCs were incubated in a hypotonic solution prior to fixation. (A–C) Fluorescent images of an OHC for prestin and di-8-ANEPPS staining. An arrowhead indicates that the plasma membrane was separated from the subsurface cisternae layer and formed a bubble that had both prestin and di-8-ANEPPS labeling. Inset in panel C is a high magnification image. Empty arrowheads indicate that the subsurface cisternae layer had only di-8-ANEPPS labeling but no prestin labeling. (D–F) Fluorescent images of an OHC for prestin labeling and di-8-ANEPPS staining. White arrows indicate that the lateral wall was ruptured by the hypotonic challenge and has neither prestin nor di-8-ANEPPS labeling. Scale bars: 10 μm ; inset: 1 μm .