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Functional expression and microdomain localization of prestin in cultured cells

Angela K. Sturm, MD¹, Lavanya Rajagopalan, PhD¹, Donald Yoo, MD¹, William E. Brownell, PhD¹, and Fred A. Pereira, PhD^{1,2}

¹ Bobby R. Alford Department of Otolaryngology–Head and Neck Surgery, Baylor College of Medicine., Houston, TX

² Huffington Center on Aging, Department of Molecular and Cellular Biology, Program in Cell and Molecular Biology, Baylor College of Medicine., Houston, TX

Abstract

INTRODUCTION—Prestin is an essential component of the molecular motor of cochlear outer hair cells that contribute to frequency selectivity and sensitivity of mammalian hearing. A model system to study prestin employs its transfection into cultured HEK 293 cells. Our goal was to characterize prestin's trafficking pathway and localization in the plasma membrane.

METHODS—We used immuno-colocalization of prestin with intracellular and plasma membrane markers and sucrose density fractionation to analyze prestin in membrane compartments. Voltage clamping was used to measure nonlinear capacitance (NLC), prestin's electrical signature.

RESULTS & DISCUSSION—Prestin targets to the membrane by 24 hours post-transfection when NLC is measurable. Prestin then concentrates into membrane foci that colocalize and fractionate with membrane microdomains. Depleting membrane cholesterol content altered prestin localization and NLC.

CONCLUSION—Prestin activity in HEK 293 cells results from expression in the plasma membrane and altering membrane lipid content affects prestin localization and activity.

Prestin is a polytopic membrane protein expressed in the lateral wall of outer hair cells (OHCs) of the mammalian cochlea.^{1–3} Prestin is a primary component of the membrane-based motor that powers OHC electromotility, which is responsible for the wide range of frequency sensitivity and selectivity in mammalian hearing.^{4,5} Prestin most likely undergoes conformational changes in response to changes in membrane potential and functions, in conjunction with the membrane and intracellular anions, to amplify the conversion of electrical signals into mechanical energy.^{5,6}

Imaging studies of the OHC lateral wall indicate that it contains structurally heterogeneous microdomain particles that have been hypothesized to contain protein molecules clustered together.^{7–9} It is also speculated that the majority of these clusters contain prestin, and that OHC electromotility is effected by small-scale conformational changes in a large number of prestin molecules in the OHC lateral wall microdomains. However, a definitive study of prestin sub-cellular localization is lacking. Due to inherent difficulties in studying prestin in outer hair cells, we have used HEK 293 cells as a model system to study prestin function.

Reprint requests: Fred A. Pereira, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030., E-mail address: E-mail: fpereira@bcm.edu.

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In the present study, we investigated the temporal expression, subcellular localization, and function of prestin in HEK 293 cells. Our results indicate that prestin is expressed in the plasma membrane, is capable of localizing to cholesterol-rich membrane microdomains, and colocalizes with membrane microdomain markers. We further show that perturbation of membrane microdomains by depletion of cholesterol affects prestin localization in membrane fractions and concomitantly alters nonlinear capacitance. We discuss the physiological significance of membrane localization of prestin, and the effect of the local membrane environment on prestin function.

MATERIALS AND METHODS

All protocols and reagents used in this study were approved by the Baylor College of Medicine Institutional Review Board.

Creation of Constructs

To create the prestin construct, we inserted a primer 5' ACCATGTACCCATACGATGTTCCAGATTACGCTCTC3' containing the hemagglutinin antigen (MYPYDVPDYAL) in-frame with the ATG start codon of the gerbil prestin cDNA (AF230376) in pBluescript (Stratagene, La Jolla, CA). The 2.7-kb *Bam*HI and *Xho*I fragment containing prestin cDNA was subcloned into the pIRES-hrGFP-1a vector (Stratagene, La Jolla, CA). This construct produces GFP as an independent protein, which marks transfected cells. The prestin-GFP construct was created by PCR amplifying a 1.6-kb gerbil prestin (AF230376) in pBluescript (above) with a high-fidelity *pfu* polymerase (Stratagene, La Jolla, CA) with a forward primer 5' GGAATTCCACCATGGATCATGCCGAAG3' and a reverse primer 5' CGGGATCCCGTGCCTCGGGTGTGGTGG3' and subsequent digestion of products with *Eco*RI and *Bam*HI for subcloning in-frame with EGFP in pEGFP-N1 vector (Clontech, Palo Alto, CA). The sequence of the regions encompassing HA-prestin and prestin-EGFP was verified.

Cell Culture and Transfections

Human embryonic kidney (HEK 293) cells were maintained with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% streptomycin and penicillin, and allowed to grow to approximately 80% to 100% confluence before passaging. Cells at confluence were passaged (1:5) and ~100,000 cells seeded on coverslips in 24-well plates. For transfections: 24 hours after passage or at ~50% confluence, the medium was replaced with fresh DMEM + 10% FBS and 20 μ L of serum-free DMEM was mixed with 0.6 μ L of FuGene6 (Roche, Indianapolis, IN), incubated for 5 minutes at room temperature; plasmid (0.2–0.4 μ g/ μ L) was added and incubated for an additional 15 minutes. DMEM with 10% FBS (500 μ L) was added and the mixture was transferred to each well dropwise before returning to culture. For electrophysiological studies, 24 hours after transfection, the cells were again trypsinized for 5 minutes using 100 μ L of 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA) at 37°C and stopped with an equal volume of FBS. The cells were pelleted (800g) and resuspended in 1 mL of DMEM + 10% FBS, and seeded onto poly-d-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA) containing 3 mL DMEM + 10% FBS at a concentration of 20,000 to 50,000 cells/dish and placed in an incubator at 37°C in 5% CO₂ overnight before nonlinear capacitance (NLC) measures.

Antibodies and Reagents

The lipid raft marker anti-Flotillin-1 (1:100) and the organelle detection sampler kit (612740) were purchased from BD Transduction Laboratories (San Jose, CA). The sampler kit included the endosome vesicle marker anti-EEA1 (1: 250), an endoplasmic reticulum marker anti-BiP/GRP 78 (1:250), a Golgi marker anti-GM130 (1:250), a plasma membrane marker anti-integrin

alpha 2/VLA-2 alpha (1: 250), and a caveolae marker anti-caveolin 1 (1:200). We also used another plasma membrane and lipid raft marker anti- $\text{Na}^+\text{K}^+\text{ATPase}$ (1:1000, Upstate Technologies, Charlottesville, VA), a clathrin vesicle marker anti-clathrin (1: 1000, Affinity Bioreagents, Golden, CO), and anti-HA poly-clonal (1:1000, Cell Signaling Technology, Danvers, MA) and anti-GFP monoclonal (Santa Cruz Biotech, Santa Cruz, CA) for immunodetection of prestin. Lastly, Alexa Fluor 594 goat anti-mouse and Vybrant Alexa Fluor 594 lipid raft labeling kit were purchased from Molecular Probes (Eugene, OR). For positive controls, the specificity for each antibody was based on detection of a single band on Western blots at the appropriate molecular mass of each protein and listed in the manufacturer's specification sheet. For negative controls, when primary antibodies were omitted in immunofluorescence detections no signals were detected. We characterized N = 8 to 10 cells for each antibody tested and all results showed little variability between cells and replicates.

Immunofluorescence and Deconvolution Microscopy

Prestin-EGFP and HA-prestin transfected cells were cultured for 48 hours before immunodetection. Cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (15 minutes). After two PBS washes (10 minutes each), the cells were permeabilized with PBT (PBS, 1% Triton X-100) for 3 minutes prior to blocking with 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (RT). Primary antibodies diluted in 1% BSA were then added and incubated for 3 hours at RT or overnight at 4°C. The cells were washed again three times in PBS (10 minutes each) before addition of secondary antibody and incubated for 1 hour at RT. Following three PBS washes (10 minutes each) the coverslips were mounted inverted on glass slides with Fluoromount antifade reagent (Molecular Probes, Carlsbad, CA) and sealed with nail polish. Images were captured on a Zeiss AxioplanII microscope with 63 × oil immersion objective with Delta Vision Applied Precision Softworx deconvolution software.

Lipid Raft Fractionation

Cell membranes were fractionated by the method of Vetrivel et al.¹⁰ Briefly, HEK 293 cells expressing prestin-EGFP or HA-prestin, treated with or without M β CD (methyl β cyclodextrin, Sigma, St. Louis, MO) for 30 minutes at 37°C before being lysed in buffer (0.5% Lubrol WX (Lubrol 17A17; Serva), 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF). Membranes were separated on a sucrose step (5%, 35%, and 45%) gradient. Twelve 1-mL fractions were collected and excess lipids removed by methanol/chloroform precipitation before the proteins were analyzed by 7.5% SDS-PAGE.

Western Blots

Proteins separated by SDS-PAGE gels were transferred to a nitrocellulose membrane. The membranes were blocked with 5% dry milk in PBS-T. Appropriate primary, followed by secondary, antibodies were used at dilutions indicated above. Bands were visualized with ECL Western blotting detection kit (Amersham, Piscataway, NJ).

Electrophysiological Measurements

Electrophysiological data were obtained from cells using the whole-cell voltage clamp technique 48 hours post-transfection. Culture dishes containing transfected cells were placed on the stage of an inverted microscope (Carl Zeiss, Gottingen, Germany). The cells were perfused with the extra-cellular solution containing Ca^{2+} and K^+ channel blockers (100 mM NaCl, 20 mM CsCl, 20 mM $\text{N}(\text{CH}_2\text{CH}_3)_4\text{Cl}$, 10 mM HEPES, 2 mM CoCl_2 , 1.47 mM MgCl_2 , 2 mM CaCl_2) prior to recording. Cells were visualized under 100× magnification oil immersion objectives (Carl Zeiss, Gottingen, Germany) and single isolated cells displaying robust GFP fluorescence (488 nm) were selected for recordings.

Patch pipettes (quartz glass) with resistances ranging from 2 to 3 MOhm were fabricated using a laser-based micropipette puller (P-2000, Sutter Instrument Company, Novato, CA). The pipettes were filled with an intracellular solution, also containing channel blockers (140 mM CsCl, 2 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES). The pH and osmolarity of both external and internal solutions were adjusted to 7.2 (± 0.02) and 300 (± 2) mOsm Kg⁻¹ with the addition of CsOH and glucose, respectively.

Cell membrane admittance, Y , was measured with the patch-clamp technique in the whole-cell mode. An electrical seal (>1 Gohm) was formed between the pipette and cell membrane, then the pipette capacitance was corrected with the compensation circuitry of an amplifier (Axon 200B, Molecular Devices, Union City, CA). Once the cell was in the whole-cell mode at 0 mV the cell admittance was monitored during a DC voltage ramp. During a ramp the voltage increased at 0.3 Vs⁻¹ from -0.16 to 0.16 V. The holding potential was 0 volts before and after the ramp. Voltages were measured relative to a Ag/AgCl reference electrode in the extracellular solution. Admittance was probed with dual-frequency stimulus.¹¹ The current, i , was measured every 10 μs and a Fast Fourier transform conducted every 1024 records to determine the real, $Re(i)$, and imaginary, $Im(i)$, parts of i every 5.12 ms at each frequency. This current was first corrected for the inherent phase shifts of the amplifier and then the $Re(Y)$ and $Im(Y)$ parts of the admittance were calculated by dividing the complex current by the complex voltage. Before an experiment, the software was calibrated for amplifier shifts by use of a 10-Mohm resistor as described.¹² A computer program written in LabView (v7.0) for Windows, in conjunction with a digital-to-analog converter card (AT-MIO-16XE-10, National Instruments, Austin, TX) controlled the calibration, stimulus, and acquisition of the admittance. The cell parameters were calculated from the admittance as described earlier.¹³ In all representations, capacitances were normalized with respect to baseline capacitance and peak capacitance, as follows:

$$C_{\text{norm}} = [C(V) - C_{\text{baseline}}] / C_{\text{baseline}}; C_{\text{final}} = C_{\text{norm}} / C_{\text{normpkc}}$$

where $C(V)$ is the calculated capacitance at voltage V , C_{baseline} is capacitance at baseline voltage (defined above), and C_{normpkc} is equal to C_{norm} at V_{pkc} .

RESULTS AND DISCUSSION

Prestin Trafficks to the Membrane and Localizes in Punctate Foci

We used deconvolution microscopy to observe prestin-EGFP fluorescence or HA-prestin immunofluorescence in HEK 293 cells from 8 to 96 hours after transfection of each construct. Prestin expression is detectable in both cases by 8 hours post-transfection, when it is seen in the endoplasmic reticulum (ER) and Golgi apparatus (Fig 1A). By 24 hours post-transfection, prestin is trafficked to the plasma membrane in most cells (Fig 1B). The punctate pattern of prestin distribution in the membrane becomes progressively more distinct after 24 hours (Fig 1C–E). At 96 hours, prestin shows high levels of expression and is found in well-defined punctate foci throughout the cell membrane (Fig 1E). Both prestin-EGFP and HA-prestin constructs showed similar trafficking and localization patterns, as evidenced by colocalization in cotransfected cells (Fig 1F; yellow fluorescence). Prestin function was readily detectable by 24 hours post-transfection, determined by nonlinear capacitance measurements of transfected cells (see below), indicating that membrane expression of prestin is necessary for function.

To confirm prestin temporal trafficking through subcellular compartments we performed immunocolocalizations. As expected for a membrane protein, prestin was detected in the characteristic pattern of the ER and Golgi by 8 hours post-transfection (Fig 1) indicating where it is synthesized and sorted for delivery to other organelles. Prestin was then detected in

endosomal vesicles as visualized by colocalization with an early endosomal marker¹⁴ (data not shown) and clathrin (Fig 2). Clathrin-coated vesicles transport proteins and lipids from the plasma membrane (endocytosis) and to internal endosome compartments.¹⁵ These vesicles are also carriers of proteins and lipids from the trans-Golgi network (TGN) to the endosome¹⁶ and may represent the normal trafficking of prestin to the plasma membrane.

Prestin Colocalizes to Plasma Membrane Microdomains

Immunofluorescence reveals that prestin colocalizes with the plasma membrane microdomain marker integrin2 α VLA-2 α , (Fig 2), but not with flotillin-1 or Na⁺/K⁺ ATPase (data not shown). Prestin also colocalizes with cholera toxin-B (CTX-B, Fig 2) that binds glycosphingolipid GM1 that cluster in lipid rafts.¹⁷ These observations, coupled with the appearance of prestin in defined foci in the membrane, indicate that a subset of prestin exists in lipid raft microdomains or that it localizes to a unique population of membrane microdomains.

To support these observations, we performed sucrose density step gradient fractionation of HEK 293 cell extracts expressing prestin. As expected, prestin was seen in higher-density fractions corresponding to ER membranes, marked by the BiP/GRP78 ER marker (Fig 3). Prestin was also detected in low-density fractions 4 and 5, which contain lipid rafts as marked by expression of flotillin-1,¹⁸ integrin2 α /VLA-2 α ,¹⁹ and Na⁺/K⁺ ATPase (Fig 3). Fractions 4 and 5 contain membrane microdomains (also known as lipid rafts, detergent-resistant membrane, or detergent-insoluble membrane) that form at the plasma membrane by clustering membrane proteins with sphingomyelin and/or cholesterol¹⁶ and are suggested to be important for modulation of signal transduction and cell adhesion and protein sorting.^{20,21}

To confirm prestin's localization in raft fractions, we treated HEK 293 cells expressing prestin with M β CD, which depletes cholesterol from membranes and prevents lipid raft formation.²² Upon cholesterol depletion with M β CD, prestin, integrin2 α /VLA-2 α , and Na⁺/K⁺ ATPase redistributed out of fractions 4 and 5 to more dense fractions (Fig 3), as expected for proteins that cluster in membrane microdomains. These biochemical and immunofluorescence data indicate prestin may localize to cholesterol-rich membrane microdomains.

Depletion of Membrane Cholesterol Alters Prestin Function

In order to study the functional significance of prestin's presence in cholesterol-rich microdomains, we measured prestin-associated NLC before and after M β CD treatment to deplete cholesterol. This voltage-dependent or nonlinear capacitance is the electrical signature of OHC electromotility.¹ HEK 293 cells expressing prestin-EGFP exhibited a bell-shaped voltage-dependent capacitance curve (24–48 hours post-transfection) with a peak (V_{pkc}) at approximately –70 mV (Fig 4). Upon cholesterol depletion with M β CD, V_{pkc} shifted towards depolarized voltages, with an average peak at +4 mV (Fig 4). All other electrophysiological characteristics of the cells, such as reversal potential and membrane resistance, remained unaltered, and the cells appeared morphologically unchanged upon cholesterol depletion. The significant alteration of prestin NLC is therefore a clear indicator that membrane cholesterol levels significantly influence prestin function.

In summary, we have shown that prestin is expressed upon transient transfection in HEK 293 cells, and is synthesized and trafficked through the endosomal secretory pathway to the cell membrane. It also associates with clathrin-coated vesicles and endosomes that shuttle proteins to and from cellular compartments, including the plasma membrane. In the membrane, prestin is capable of localizing to cholesterol-rich microdomains and depleting cholesterol from the cell causes alterations in the membrane distribution of prestin, moving it out of lipid raft fractions. Importantly, depletion of cholesterol affects prestin function in HEK 293 cells. The physiological significance of this observation needs to be further tested directly in OHCs.

Determining normal cholesterol levels in the native membrane environment of prestin in these cells, and testing the effect of raising or lowering cholesterol in the OHC lateral wall, will yield further information on the physiological role of membrane microdomains in prestin function.

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Figure 1.

Temporal expression and membrane localization of prestin in HEK 293 cells. Prestin was expressed from two separate constructs, prestin-GFP and HA-prestin, in HEK 293 cells. Cellular localization of prestin was detected by immunofluorescence to the HA tag (red) or epifluorescence of prestin-EGFP (green) and images captured by deconvolution microscopy. Both prestin-EGFP and HA-prestin are expressed by 8 hours post-transfection (**A**), are seen in the plasma membrane by 24 hours (**B**), and show progressively less uniform and more punctate distribution by 24 to 96 hours (**C–E**). In addition, both prestin-EGFP and HA-prestin colocalize as evinced by overlay (yellow) of their fluorescence signals (**F**).

Figure 2.

Subcellular localization of prestin. Prestin trafficks through the Golgi and endoplasmic reticulum (data not shown) as expected before extensively colocalizing to clathrin vesicles and to endosomes. Prestin is seen to colocalize to the plasma membrane with integrin2 α /VLA-2 α and lipid raft marker CTX-B, which binds GM1 glycosphingolipids. Colocalization is visualized as yellow fluorescence (arrowheads) due to overlap of red and green fluorescence. Images were captured using a Zeiss Axioplan II deconvolution microscope with 63 $\times$ oil immersion objective.

Figure 3.

Prestin localizes with microdomain markers. Prestin colocalizes in lanes 4/5 with flotillin-1, a raft localized protein, and membrane proteins Na⁺/K⁺ATPase and integrin2α/VLA-2α. All prestin-EGFP in lane 4/5 redistributes into higher-density fractions containing ER membranes (BiP/GRP 78 positive) after depletion of cholesterol with MβCD.

Figure 4.

Cholesterol depletion causes a shift in peak capacitance in prestin NLC. Prestin-EGFP transfected cells were treated with M β CD for 30 minutes at 37°C to deplete cholesterol before being patched to measure nonlinear capacitance. The peak of the capacitance shows a significant shift towards depolarizing voltages (*arrow*). The average voltage at maximum capacitance was $-71.25 \text{ mV} \pm 13.6 \text{ mV}$, $n = 4$ (untreated), and $10.25 \text{ mV} \pm 8.7 \text{ mV}$, $n = 4$ (cholesterol depleted). The electrophysiological characteristics of the cells (membrane resistance, reversal potential, etc.) remained unaltered.