Lack of pendrin HCO$_3^-$ transport elevates vestibular endolymphatic [Ca$^{2+}$] by inhibition of acid-sensitive TRPV5 and TRPV6 channels

Kazuhiro Nakaya,1,4 Donald G. Harbidge,1 Philine Wangemann,2 Bruce D. Schultz,3 Eric D. Green,5 Susan M. Wall,6 and Daniel C. Marcus1

1Cellular Biophysics Laboratory, 2Cell Physiology Laboratory, and 3Epithelial Cell Biology Laboratory, Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas; 4Department of Otolaryngology-Head and Neck Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan; 5Genome Technology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland; and 6Renal Division, Emory University School of Medicine, Atlanta, Georgia

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Pendrin (PDS or SLC26A4) is an anion exchanger that is capable of transporting iodide, chloride, formate, nitrate, and bicarbonate and functions as an HCO$_3^-$-secreting mechanism in kidney (30, 31, 33). SLC26A4 is expressed in kidney (29), the inner ear, thyroid (7), mammary gland (26), uterus (34), testis (16), vas deferens (3), and placenta (2). Mutation or deletion of SLC26A4 gene leads to acidification of the urine (15) and defects that occur in the absence of SLC26A4 (6). SLC26A4 knockout mice exhibit reduced pH and [Ca$^{2+}$] in the apical membrane of vestibular transitional cells in the utricle and ampullae (40). If SLC26A4 secretes HCO$_3^-$, which in turn would lead to an elevation of luminal pH and [Ca$^{2+}$] in the vestibule and if there are no strong compensatory mechanisms, it can be predicted that there may be an acidification of endolymph when SLC26A4 is deleted. An altered endolymphatic pH can be expected to affect other ion transport processes in the luminal membrane of epithelial cells bordering the lumen, since several ion channels are known to be highly sensitive to extracellular pH.

The Ca$^{2+}$ concentration ([Ca$^{2+}$]) of vestibular endolymph (~250 μM) is lower than that of perilymph (~1 mM), and it has a critical role in sensory transduction through hair cells (20). A Ca$^{2+}$ absorption system in inner ear epithelial cells must be present to maintain the low [Ca$^{2+}$] of vestibular endolymph. Recently, our group reported that the epithelial Ca$^{2+}$ channels TRPV5 and TRPV6 (transient receptor potential types 5 and 6) are expressed in the vestibular system and that TRPV5 expression is stimulated by 1,25-dihydroxyvitamin D$_3$, as also reported in kidney. TRPV5/6 channels are known to be inhibited by extracellular acidic pH. Endolymphatic pH, [Ca$^{2+}$], and transepithelial potential of the utricle were measured in Cl$^-$/HCO$_3^-$ exchanger knockout mice (SLC26A4) and Slc26a4−/− mice exhibit reduced pH and utricular endolymphatic potential and increased [Ca$^{2+}$]. Monolayers of primary cultures of rat semicircular canal duct cells were grown on permeable supports, and cellular uptake of 45Ca$^{2+}$ was measured individually from the apical and basolateral sides. These observations support the notion that one aspect of vestibular dysfunction in Pendred syndrome is a pathological elevation of endolymphatic [Ca$^{2+}$] due to luminal acidification and consequent inhibition of TRPV5/6-mediated Ca$^{2+}$ absorption.

Address for reprint requests and other correspondence: D. C. Marcus, Kansas State Univ., Dept. of Anatomy and Physiology, 228 Coles Hall, Manhattan, KS 66506-5802 (e-mail: marcus@ksu.edu).

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We indeed observed a reduced pH and increased \([\text{Ca}^{2+}]\) in the vestibular lumen of \(\text{Slc26a4}^{-/-}\) mice. Furthermore, 1.25\((\text{OH})_2\)\(\text{D}_3\) increased \(\text{Ca}^{2+}\) absorption and TRPV5/6 inhibitors reduced apical uptake of \(\text{Ca}^{2+}\). \(\text{Ca}^{2+}\) absorption was inhibited by apical acid pH and was stimulated by apical alkaline pH, consistent with the notion that one aspect of vestibular dysfunction in Pendred syndrome is a pathological elevation of endolymphatic \([\text{Ca}^{2+}]\) due to luminal acidification and consequent inhibition of TRPV5/6-mediated \(\text{Ca}^{2+}\) absorption.

**METHODS**

**Utricular endolymphatic potential, pH, and \([\text{Ca}^{2+}]\).** Adult \(\text{Slc26a4}^{-/-}\) and \(\text{Slc26a4}^{+/-}\) mice were obtained from a colony at Kansas State University that was established with breeders kindly provided by Dr. Susan Wall. The mouse strain 129Sv/Ev (Taconic, Germantown, NY) was used as the source of \(\text{Slc26a4}^{-/-}\) mice, since \(\text{Slc26a4}^{-/-}\) mice were generated in this background. Young adult mice 30–142 days old were deeply anesthetized with tribromoethanol (640 mg/kg ip; Fluka 90710) in 0.9% NaCl. The Institutional Animal Care and Use Committee of Kansas State University approved all experimental protocols.

We measured utricular endolymphatic potential (UP), pH, and \([\text{Ca}^{2+}]\) with double-barreled microelectrodes (one side pH or \(\text{Ca}^{2+}\) sensitive and the other voltage sensitive; see below for details) using procedures developed by modifying previously described protocols (18, 19). For both pH and \(\text{Ca}^{2+}\) electrodes, two pieces of glass tubing containing a glass filament (World Precision Instruments 1B100F-4, Sarasota, FL) were cut to 81 mm and 60 mm and pulled with a micropipette puller (Narishige PD-5, Tokyo, Japan). After pulled capillaries were heated at 180°C for 2 h, the ion-selective barrel was silanized by placing the open end of that barrel through a hole in the lid of a beaker at 210°C in which we put 0.08 ml of dimethylidichlorosilane (Fluka 40136) for 90 s. The reference barrel was protected from silanization by sealing the open end with Parafilm (Alcan Packaging). After electrodes were heated at 180°C for 3 h, the tips were broken to ~3-μm outer diameter.

For pH electrodes, the reference barrel was filled with 1 M KCl, and the ion-selective barrel was filled at the tip with hydrogen ionophore II-cocktail A (Fluka 95297) and back filled with buffer solution (500 mM KCl, 20 mM HEPES, pH 7.34). For \(\text{Ca}^{2+}\) electrodes, the reference barrel was filled with 150 mM KCl, and the ion-selective barrel was filled at the tip with calcium ionophore I-cocktail A (Fluka 21048) and back filled with 500 mM CaCl\(_2\). Connection to each barrel was made with a Ag AgCl wire. Each electrode was connected to an input of a dual electrometer (World Precision Instruments FD223), and buffered outputs were led to a data acquisition system (Axon Instruments DIGIDATA 1322A) and recorded with AxoScope 9 (Axon Instruments). A pulse generator was used to inject current pulses (1 nA) through the reference barrel to monitor the resistance of the electrode.

pH electrodes were calibrated at three different pH values (composition in mM): pH 6 (130 NaCl, 20 MES), pH 7 (130 NaCl, 20 HEPES), and pH 8 (130 NaCl, 20 tricine) and had an average slope of 56.6 ± 0.7 mV/pH (n = 16). \(\text{Ca}^{2+}\) electrodes were calibrated at three \(\text{Ca}^{2+}\) concentrations (composition in mM): 10 μM \(\text{Ca}^{2+}\) [0.12 mM CaCl\(_2\), 150 KCl, 10 HEPES, 1 Ca\(_2\)NO\(_3\)Na\(_2\) (nitrilotriacetic acid; Sigma N-0128), pH 7.4], 100 μM \(\text{Ca}^{2+}\) (0.1 CaCl\(_2\), 150 KCl, 10 HEPES, pH 7.4), and 1 mM \(\text{Ca}^{2+}\) (1 CaCl\(_2\), 150 KCl, 10 HEPES, pH 7.4) and had an average slope of 26.2 ± 0.8 mV/decade concentration (n = 13). Data were analyzed with custom software written by P. Wangemann in LabTalk (Origin 6.0; OriginLab, Northampton, MA).

To reduce the diffusion of CO\(_2\) through the exposed surface of the perilymph to the ambient air, which leads to higher pH than in unexposed perilymph, we put liquid Sylgard 184 (Dow Corning, Midland, MI) at the fluid surface after placing the electrodes in the perilymph. The electrode was maintained below the surface of the perilymph solution after measurements were made of inner ear fluids, and the first calibration point was taken in situ in the bulla pocket of the temporal bone. The pH 7 buffer for calibration was placed on the Sylgard layer, and the electrode was retracted up into this buffer from the perilymph surrounding the utricle. Approximately 85% of the electrodes survived the travel through the Sylgard layer; outcomes from experiments in which electrodes failed this maneuver were excluded from the data set. The remaining calibration points to determine slope sensitivity were obtained by placing calibration solutions in a small-conducting Ringer-agarose (BP160; Fisher Scientific) cup that rested on the exposed neck muscles. This procedure was used to preclude voltage offsets introduced through movement of the reference electrode. In some cases, the electrode failed after moving to the calibration cup. In these few cases, the preexperiment electrode slope was used in conjunction with the in situ calibration point. Calibration of \(\text{Ca}^{2+}\) electrodes was identical except that the first point was taken by flushing the perilymph space with 1 mM \(\text{Ca}^{2+}\) solution and raising the electrode into a region of the pool remote from the tissues.

Perilymph pH, \([\text{Ca}^{2+}]\), and UP were corrected for the liquid junction potentials of 1.3 mV (pH electrode) and 4 mV (\(\text{Ca}^{2+}\) electrode) between the voltage barrels in calibrating solution and in perilymph and between the voltage electrode in perilymph and endolymph.

**Cellular uptake measurements.** Primary cultures of Wistar rat SCCD were prepared and incubated in DMEM-F-12 (12500-062; Invitrogen) supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), as described previously (44). Primary cultures of SCCD cells from rats were used to study transport function because attempts to proliferate murine SCCD cells in culture were unsuccessful. Briefly, SCCD epithelial cells from neonatal rats were cultured on 12-mm-diameter Snapwell permeable supports (0.4-μm pore size, Costar 3801, Corning, NY). Confluence of primary cultures (10–14 days after seeding) was verified visually and by measurement of transepithelial electrical resistance using and Endohm meter (World Precision Instruments). Cultures were incubated further for 24 h in the presence or absence of 1,25\((\text{OH})_2\)\(\text{D}_3\) (DM-200; Biomol, Plymouth Meeting, PA) (44).

The method for cellular \(45\text{Ca}^{2+}\) uptake determination was modified from Den Dekker et al. (5). Cells were washed twice on both sides with prewarmed \(\text{Ca}^{2+}\)-free solution (in mM: 145 NaCl, 4 KCl, 1 MgCl\(_2\), 10 HEPES, 5 glucose, pH 7.4). Cells were then incubated in this medium at 37°C and 5% CO\(_2\) for 45–60 min before treatment. Cells tested with \(\text{Ca}^{2+}\) channel blockers or different pH buffers had the apical medium changed after 45 min of pretreatment, allowing 15-min incubation with the appropriate blocker or pH. The blocker medium was \(\text{Ca}^{2+}\)-free solution with an added mixture of known inhibitors of TRPV5/6 (10 μM ruthenium red, 100 μM LaCl\(_3\), and 100 μM GdCl\(_3\) or each blocker in the same concentration by itself. The various pH buffers were similar to \(\text{Ca}^{2+}\)-free solution except that the pH 5.5 contained 20 mM MES as a buffer in place of HEPES and the pH of each was adjusted with HCl or NaOH as appropriate. The pretreatment buffer on either the apical or basolateral side was replaced with \(45\text{Ca}^{2+}\) uptake buffer (in mM: 145 NaCl, 4 KCl, 1 MgCl\(_2\), 0.1 CaCl\(_2\), 20 HEPES, 5 glucose, pH 7.4), and at least 1 μCi \(45\text{Ca}^{2+}\) (6.8 GBq/mCi) for 15 min. Verapamil was utilized to preclude measuring \(\text{Ca}^{2+}\) uptake via L-type \(\text{Ca}^{2+}\) channels, which play important roles in some epithelia (27). Verapamil at the concentration used here is sufficient for a virtually complete block of \(\text{Ca}^{2+}\) current through these channels (13). The radiotracer was purchased as 1 μCi (37 MBq) \(45\text{Ca}^{2+}\) as CaCl\(_2\) in 17 μl of H\(_2\)O (NEZ013001MC; Perkin-Elmer Life Sciences, Boston, MA).

The cells were then rinsed three times on both sides with ice-cold wash buffer (in mM: 145 NaCl, 4 KCl, 1 MgCl\(_2\), 0.5 CaCl\(_2\), 1.5 LaCl\(_3\), 0.7 mGdCl\(_3\), and 20 HEPES, pH 7.4).
20 HEPES, 5 glucose, pH 7.4). The wash buffer was removed from both compartments, and 200 μl of lysis buffer (lysis reagent 1 from cAMP kit RPN225; Amersham Biosciences, Piscataway, NJ) were added to the apical compartment. After 5 min, the lysate was collected and radioactivity was determined with a liquid scintillation counter (Packard Tri-Carb 2100TR, Meriden, CT). The difference in uptake of $^{45}\text{Ca}^{2+}$ from the apical and basolateral sides was taken as a relative measure of net flux.

Statistics. Data are presented as means $\pm$ SE from $n$ observations. Net uptakes are the difference of the mean apical and basolateral uptakes; the SE was pooled from the variance of the apical and basolateral uptake data, as calculated from the variances with the Java applet at http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Pooled.htm. Significance between the UP, pH, and $\text{Ca}^{2+}$ control and knockout mouse data and the significance of $\text{Ca}^{2+}$ uptake data were calculated with an unpaired $t$-test. Comparison of endolymph values to the corresponding perilymph values (see Fig. 2) was performed with paired $t$-test. $P < 0.05$ represents a significant difference.

RESULTS

UP, pH, and $[\text{Ca}^{2+}]$ in the utricle. Across all measurements, Slc26a4$^{-/-}$ mice showed no significant differences in UP, pH, and $[\text{Ca}^{2+}]$ compared with Slc26a4$^{+/+}$ mice, consistent with an autosomal recessive trait. There were no differences between Slc26a4$^{+/+}$ mice compared with Slc26a4$^{-/-}$ mice in UP $[-1.5 \pm 0.5 \text{ mV (n = 8)}$ vs. $-1.5 \pm 0.3 \text{ mV (n = 9)})$, perilymphatic pH $[7.14 \pm 0.02 (n = 9)$ vs. $7.19 \pm 0.02 (n = 5)]$, and endolymphatic pH $[7.42 \pm 0.02 (n = 5)$ vs. $7.40 \pm 0.06 (n = 9)].$
(n = 5), perilymphatic [Ca\(^{2+}\)] [1.62 ± 0.10 mM (n = 3) vs. 1.51 ± 0.28 mM (n = 4)], endolymphatic [Ca\(^{2+}\)] [266 ± 51 \(\mu\)M (n = 3) vs. 230 ± 28 \(\mu\)M (n = 4)]. The data and the SE were pooled between the two genotypes and taken as “control” reference values against which the knockout mice were evaluated.

There was a small, but statistically significant, decrease of ~3 mV in the UP of Slc26a4\(^{-/-}\) mice compared with control mice (Fig. 1). No significant differences between the control and knockout mice were observed in perilymphatic pH and [Ca\(^{2+}\)] (Fig. 1). The pH was higher in endolymph of control mice than in the perilymph, consistent with secretion of HCO\(_3^-\) by pendrin. Endolymphatic pH was significantly decreased in Slc26a4\(^{-/-}\) mice compared with control mice (Fig. 2). Furthermore, endolymphatic [Ca\(^{2+}\)] was less than in perilymph of control mice and was significantly increased in Slc26a4\(^{-/-}\) mice to a level higher than in perilymph (Fig. 2). These abnormalities of endolymphatic potential, pH, and [Ca\(^{2+}\)] in Slc26a4\(^{-/-}\) mice are compatible with their vestibular dysfunction, exhibited as circling behavior and head tilting (6).

The high endolymphatic [Ca\(^{2+}\)] in Slc26a4\(^{-/-}\) mice would be consistent with altered otoconia, as reported earlier (41). Indeed, we consistently observed an absence of the normal otoconia but observed a single giant crystal (Fig. 3), assumed to be CaCO\(_3\), which is the compound of calcium observed in otoconia.

**Increased Ca\(^{2+}\) absorption by 1,25(OH\(_2\))\(_D_3\).** We tested whether Ca\(^{2+}\) absorption is stimulated by exposure to 1,25(OH\(_2\))\(_D_3\), since transcript expression of two genes involved in epithelial Ca\(^{2+}\) absorption, TRPV5 and calbindin, is upregulated (44). Stimulation of transport would therefore serve as part of the fingerprint for the involvement of this Ca\(^{2+}\) transport system in vestibular function.

Apical-to-cell \(^{45}\)Ca\(^{2+}\) uptake and basolateral-to-cell \(^{45}\)Ca\(^{2+}\) uptake in primary SCCD cells were measured separately. Net uptake was obtained by subtraction of the mean basolateral-to-cell \(^{45}\)Ca\(^{2+}\) uptake from the mean apical-to-cell \(^{45}\)Ca\(^{2+}\) uptake. Without any added inhibitors of TRPV5/6, cultured cells exhibited a net \(^{45}\)Ca\(^{2+}\) uptake consistent with an absorptive flux from the apical to the basolateral side (Fig. 4). This direction corresponds to endolymph (normally, low [Ca\(^{2+}\)]) to perilymph (normally, high [Ca\(^{2+}\)]). Incubation with 1.25(OH\(_2\))\(_D_3\) for 24 h significantly increased net Ca\(^{2+}\) uptake by 71% (Fig. 4).

**Regulation by pH.** The pH of the apical solution was varied over a wide range to test the dependence of Ca\(^{2+}\) uptake on apical pH of the SCCD cells. Decreases of the apical pH from 8.2 to 7.4 and 5.5 each significantly reduced net \(^{45}\)Ca\(^{2+}\) uptake by 37 and 96%, respectively (Fig. 5).

**Inhibition of Ca\(^{2+}\) uptake by inhibitors.** Adding a mixture of agents known to block TRPV5 and TRPV6 Ca\(^{2+}\) channels (in \(\mu\)M: 10 ruthenium red, 100 LaCl\(_3\), 100 GdCl\(_3\)) stopped the net uptake completely (Fig. 6A). Both apical-to-cell and basolateral-to-cell uptakes were significantly reduced (Fig. 6A). Because the mixture of Ca\(^{2+}\) inhibitors completely blocked Ca\(^{2+}\) net uptake, individual blockers were examined.

![Fig. 3. Otoconia from control (Slc26a4\(^{-/-}\)) and knockout (Slc26a4\(^{-/-}\)) mice. A: normal otoconia situated in utricle from Slc26a4\(^{-/-}\) mouse. B: normal otoconia isolated from Slc26a4\(^{-/-}\) utricle at higher magnification. C: utricle from Slc26a4\(^{-/-}\) mouse with giant crystal (arrow). D: giant crystal isolated from Slc26a4\(^{-/-}\) utricle at higher magnification.](http://ajprenal.physiology.org/)
Fig. 4. Upregulation of \(^{45}\text{Ca}^{2+}\) uptake by 1,25-dihydroxyvitamin D\(_3\) \(\text{[1,25(OH)\textsubscript{2}D\textsubscript{3}]}\) across semicircular canal duct (SCCD) cells grown on permeable supports. \textit{Left}: apical-to-cell \(^{45}\text{Ca}^{2+}\) uptake (apical) and basolateral-to-cell \(^{45}\text{Ca}^{2+}\) uptake (basolateral) of cells incubated with and without 1,25(OH)\(_{2}\)D\(_{3}\) (Vit D, 100 nM, 24 h). \textit{Right}: net \(^{45}\text{Ca}^{2+}\) uptake was calculated by subtracting mean basolateral-to-cell \(^{45}\text{Ca}^{2+}\) uptake from mean apical-to-basolateral \(^{45}\text{Ca}^{2+}\) uptake; SE values were pooled. Net \(^{45}\text{Ca}^{2+}\) uptake of cells with 1,25(OH)\(_{2}\)D\(_{3}\) was regarded as 100% \((n = 4)\). Uptakes are expressed as counts per minute (cpm). *\(P < 0.05\).

Ruthenium red (10 \(\mu\text{M}\)) significantly decreased net \(\text{Ca}^{2+}\) uptake by 31% (Fig. 6B); 100 \(\mu\text{M}\) LaCl\(_3\) and 100 \(\mu\text{M}\) GdCl\(_3\) showed stronger effects than ruthenium red (Fig. 6C).

**DISCUSSION**

We report for the first time that \(\text{Sle26a4}^{-/-}\) mice possess a markedly lower pH and higher \([\text{Ca}^{2+}]\) in the luminal fluid, endolymph, than wild-type and heterozygous mice. We also present the first evidence of net \(\text{Ca}^{2+}\) absorption by SCCD epithelium in the vestibular system.

\(\text{Sle26a4}\) is expressed in several discrete areas in the inner ear, including the apical membrane of vestibular transitional cells of the utricle and ampullae of the semicircular canals (8, 40). These observations, along with the absence of pendrin transcript in the SCCD (N. Raveendran and D. Marcus, unpublished observations), suggest that pH homeostatic contributions by the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger pendrin occurs mainly outside of the SCCD.

Despite the physical separation between the sites of HCO\(_3\)\(^-\) secretion and of calcium absorption, it is expected that there is no significant gradient of pH among the utricle, ampullae, and canal ducts since the lumen has a relatively large cross-sectional area that favors good diffusion, unlike most smaller tubular epithelial structures such as the nephron. The volume of the utricle is almost as large as the SCCD (32), and convection of endolymph through the SCCD is one of the mechanisms of vestibular perception (14, 39). It is thus reasonable to assume that both the utricle and the SCCD have similar ion concentrations and pH by diffusion and convection. The TRPV5/6 channels in the SCCD would therefore be exposed on their extracellular face to the relatively acidic conditions in the \(\text{Sle26a4}^{-/-}\) mice.

Regulation of luminal pH by \(\text{Sle26a4}\) could conceivably influence transport of a number of ions that are actively moved by vestibular epithelial cells. However, the deletion of \(\text{Sle26a4}\) had no effect on luminal (endolymphatic) K\(^+\) concentration in the utricle (28), suggesting that neither the K\(^+\) secretory channel in the apical membrane of vestibular dark cells nor K\(^+\) exit pathways are strongly sensitive to luminal pH. However, the negative shift of the UP in \(\text{Sle26a4}^{-/-}\) mice is consistent with an increase in apical membrane conductance of highly polarized epithelial cells, such as the hair cells (17), as opposed to low-voltage K\(^+\)-secretory dark cells (41). Increased apical conductance would increase the fractional contribution of the negative intracellular potential to the observed transepithelial potential, the UP (17). A candidate for the apical acid-activated conductance is the ASIC1b channel identified in the apical membrane of vestibular hair cells (35), although this channel in an oocyte expression system was only transiently activated by acid. ASIC1b inactivated during exposure to a step change in acid with a time constant on the order of 1 s (1).

Previously, we reported the expression of the epithelial \(\text{Ca}^{2+}\) channels TRPV5 and TRPV6 in primary cultures of SCCD epithelial cells (44). Because of the known sensitivity of the TRPV5 and TRPV6 channels to external pH (46), we hypothesized that low pH of vestibular endolymph due to absence of the Cl\(^-\)/HCO\(_3\)\(^-\) exchanger pendrin inhibits TRPV5 and TRPV6 and consequently leads to higher \([\text{Ca}^{2+}]\) in endolymph.

The normal \([\text{Ca}^{2+}]\) of vestibular endolymph (\(\sim 250 \mu\text{M}\)) (42) is known to be necessary for optimal hair cell function (20) and is likely important in the utricle for proper otoconial formation, which contains precipitated CaCO\(_3\). The otoconial crystals comprise the inertial mass that is coupled to the sensory stereocilia of the utricular hair cells and are relatively fine “stones” under normal conditions but form giant crystals in \(\text{Sle26a4}^{-/-}\) mice (Fig. 3) (40). The low \([\text{Ca}^{2+}]\) of normal endolymph points to the existence of one or more \(\text{Ca}^{2+}\) absorption pathway in the vestibular system. However, in

Fig. 5. Net \(^{45}\text{Ca}^{2+}\) uptake under different apical pH levels. Measurements were made after a 15-min incubation with pH 5.5, 7.4, and 8.2. All cells were incubated with 1,25(OH)\(_{2}\)D\(_{3}\) (100 nM, 24 h) before measurement \((n = 6)\).
addition to absorption, it is known that there are also Ca$^{2+}$ secretory processes in the inner ear. The plasma membrane Ca$^{2+}$-ATPase isofrom 2 is expressed in the apical membrane (sterocilia) of hair cells, and mutation of this isoform leads to an absence of otoconia, a lowered Ca$^{2+}$ concentration of the stereocilia of hair cells, and mutation of this isoform leads to vestibular dysfunction (43).

Our results here of elevated Ca$^{2+}$ concentration in the endolymph of Scl26a4$^{-/-}$ mice compared with the endolymph of wild-type mice is consistent with inhibition of the Ca$^{2+}$-absorbing function of the TRPV5 and TRPV6 channels by acidic pH in those mice. Interestingly, endolymphatic Ca$^{2+}$ was also higher than the abluminal fluid, perilymph, in Scl26a4$^{-/-}$ mice. That elevation of endolymphatic Ca$^{2+}$ (2.6 mM compared with 1.1 mM in perilymph) is even higher than the equilibrium concentration of 1.5 mM that could be accounted for by passive distribution of Ca$^{2+}$ at the UP of $-4.4$ mV in knockout mice. These observations point to continued active secretion of Ca$^{2+}$ in the vestibular system in the face of inhibited Ca$^{2+}$ absorption.

TRPV5 is predominantly expressed and controlled by 1,25(OH)$_2$D$_3$ in the kidney, whereas TRPV6 is predominantly expressed and controlled by 1,25(OH)$_2$D$_3$ in the intestine (9, 36). Although the SCCD epithelium expresses transcripts for both TRPV5 and TRPV6, only TRPV5 is responsive to 1,25(OH)$_2$D$_3$ in SCCD primary cultures (44) and expression of TRPV6 is $\sim 10$-fold less in the native epithelium (D. Yamauchi, N. Raveendran, D. Marcus; unpublished observations). Our observation of increased Ca$^{2+}$ absorption by SCCD is consistent with observations in kidney and with the increased expression of TRPV5 transcript by 1,25(OH)$_2$D$_3$ in SCCD.

Both intra- and extracellular acid pH inhibit TRPV5, and they each enhance the other’s effect (45). Binding of extracellular protons to glutamate 522 in the pore region of TRPV5 causes conformational changes and closure of the channel (46). TRPV6 has a histidine at the position equivalent to glutamate 522 in TRPV5 which predicts similar pH sensitivity for both TRPV5 and TRPV6 (46). The very steep dependence of activity of these channels and of SCCD Ca$^{2+}$ uptake on pH is strong evidence for the functional expression of the TRPV5 and TRPV6 channels in the apical membrane of SCCD epithelium.

In addition, TRPV5 is known to be inhibited by ruthenium red, gadolinium, and lanthanum (12, 38). TRPV6 is also inhibited by ruthenium red but with less potency [IC$_{50}$ of 0.12 $\mu$M rabbit TRPV5 vs. 9 $\mu$M mouse TRPV6 (12)]. Each of the multivalent blockers has poor specificity, but the effectiveness of all of these conditions to decrease Ca$^{2+}$ absorption in SCCD epithelium strongly supports the notion that a major part of the Ca$^{2+}$ absorption from the semicircular canal is via the TRPV5 and TRPV6 channels. Ruthenium red is an inhibitor of a wide range of Ca$^{2+}$ channels, including voltage-gated and epithelial
Ca\(^{2+}\) channels (4, 12). The net Ca\(^{2+}\) uptake in the SCCD was inhibited only 31\% by 10 \(\mu\)M ruthenium red despite the 100\% lower IC\(_{50}\) mentioned above for TRPV5 and IC\(_{50}\) for TRPV6 similar to the concentration used. This result suggests either that there is a significant contribution of another Ca\(^{2+}\)-permeable channel in this epithelium or, more likely, points to a combination of species and condition differences to the cited studies.

The trivalent cations La\(^{3+}\) and Gd\(^{3+}\) are also effective blockers of TRPV5 and TRPV6 (23, 38) but are also not specific enough to discriminate TRPV5 and TRPV6 from other Ca\(^{2+}\) channels. In our study, a mixture of ruthenium red, La\(^{3+}\), and Gd\(^{3+}\) produced a complete block of net Ca\(^{2+}\) uptake.

Vectorial Ca\(^{2+}\) absorption occurs by well-characterized processes (10). Ca\(^{2+}\) enters the cell from the lumen down an electrochemical gradient through TRPV5 and TRPV6 in the apical cell membrane. Cytotoxic accumulation of Ca\(^{2+}\) in the cytosol is prevented by immediate binding of Ca\(^{2+}\) to Ca\(^{2+}\)-binding proteins, calbindin-9K, and/or calbindin-28K, which carry Ca\(^{2+}\) in a bound state by diffusion to the basolateral cell membrane. Ca\(^{2+}\) is then extruded from the cell by an ATP-dependent plasma membrane Ca\(^{2+}\)-ATPase and an Na\(^{+}/Ca\(^{2+}\) exchanger. In this way, influx at the apical membrane and apical-to-basolateral flux are correlated in a 1:1 fashion (24).

The radiotracer accumulation in the cells from the basolateral side predominantly indicates the basolateral-to-cell unidirectional flux of the Na\(^{+}/Ca\(^{2+}\) exchanger. As the net flux is reduced, this coupled basolateral uptake will also be reduced, as seen in Fig. 6.

In conclusion, we have demonstrated an acid-sensitive vectorial Ca\(^{2+}\) absorption by the SCCD that can account for the pathological endolymphatic composition observed in mice with the SLC26A4 mutation. These observations increase our understanding of the etiology of Pendred syndrome.

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