The extracellular loop of pendrin and prestin modulates their voltage-sensing property

Makoto F. Kuwabara1, Koichiro Wasano2, Satoe Takahashi2, Justin Bodner3, Tomotaka Komori1, Sotaro Uemura1, Jing Zheng2,4, Tomohiro Shima1,2, and Kazuaki Homma2,4,*

1Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, JAPAN, 2Department of Otolaryngology – Head and Neck Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA, 3DePaul University, Chicago, IL 60614, USA, 4The Hugh Knowles Center for Clinical and Basic Science in Hearing and Its Disorders, Northwestern University, Evanston, IL 60608, USA

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*Address correspondence to: Kazuaki Homma, 303 E Chicago Ave, Chicago, IL 60611, USA. Tel: +1-312-503-5344, E-mail: k-homma@northwestern.edu; Tomohiro Shima, Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, JAPAN. Tel: +81-03-5841-4399, E-mail: tomohiro.shima@bs.s.u-tokyo.ac.jp

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ABSTRACT

Pendrin and prestin belong to the solute carrier 26 (SLC26) family of anion transporters. Prestin is unique among the SLC26 family members in that it displays voltage-driven motor activity (electromotility) and concurrent gating currents that manifests as nonlinear cell membrane electrical capacitance (NLC). Although the anion transport mechanism of the SLC26 proteins has begun to be elucidated, the molecular mechanism of electromotility, which is thought to be evolved from an ancestral ion transport mechanism, still remains largely elusive. Here, we demonstrate that pendrin also exhibits large NLC, and that charged residues present in one of the extracellular loops of pendrin and prestin play significant roles in setting the voltage-operating points of NLC. Our results suggest that the molecular mechanism responsible for sensing voltage is not unique to prestin among the members of the SLC26 family, and that this voltage-sensing mechanism works independently of the anion transport mechanism.

The solute carrier 26 (SLC26) proteins are ubiquitously expressed in a wide variety of species from bacteria to humans, and constitute the SLC26/SulP family of ion transporters and channels (1). While most of the members of this large gene family function as anion exchangers or channels, only prestin (SLC26A5) exhibits voltage-dependent motor activity referred to as electromotility. Electromotility of prestin and the anion transport mediated by pendrin (SLC26A4) are essential for normal development and operation of mammalian inner ears (2-4). Prestin-mediated electromotility confers a rapid voltage-induced force-generating cell length change on cochlear outer hair cells (5), which is indispensable for the frequency selectivity and sensitivity of mammalian hearing (4). Recent structural and biochemical studies (6,7) have provided significant insights into the ion transport mechanism of the SLC26 proteins. However, the molecular mechanism of the voltage-driven motor activity, which is thought to be unique to prestin among the SLC26 family members, still remains largely unknown. Electromotility accompanies a movement of prestin-associated charge. Since this charge movement is rapid, it manifests as nonlinear electrical capacitance of the cell membrane (nonlinear capacitance, NLC) (8,9). Thus, NLC is regarded as the electric signature of
Pendrin retains voltage-sensing ability. Its electromotility, and its measurement often substitutes for direct electromotility measurement.

Due to the high degree of similarity in amino-acid sequences among the SLC26 family members, it is conceivable that the tertiary structures of SLC26 proteins are quite similar among the family members, and that a common molecular mechanism underlies their diverse physiological functions. Both pendrin and prestin are expressed in the mammalian inner ear, and they share 36% homology at the amino acid level. Using SLC26 models that were generated based on the solved structure of a bacterial SLC26 protein (Fig. 1) (7), we carried out a comprehensive mutational analysis combined with whole-cell patch-clamp recordings and anion transport assays to understand the molecular basis for the functional difference between pendrin, an well-characterized anion transporter, and prestin, a voltage-driven motor. We found that pendrin also retains voltage-sensing ability, which becomes evident with addition of cyclodextrin to depolarize the peak voltage operating point. We also found that the electrostatic property of the characteristic extracellular loop of pendrin and prestin (termed “L2” in this study, Fig. 1) influences the voltage operating points of these proteins. Furthermore, the anion transport activity of pendrin was not affected by the alterations in operating voltage range, suggesting that the voltage-sensing and anion transport mechanism are independent of each other. Our results suggest that the extracellular loop of prestin has adopted the optimal electrostatic property, which affects the membrane surface electric potential, so that the voltage-operating point resides within the physiologically relevant voltage range, and that the electromotility may have evolved independently of its anion transport mechanism.

RESULTS

The extracellular loops of pendrin and prestin

Recent structural and biochemical studies provided significant insights into the ion transport mechanism of the SLC26 proteins (6,7,10). Since the basic architecture of the transmembrane (TM) region that accommodates the putative ion translocation pathway is likely quite similar among the SLC26 family members, it is possible that the diverse physiological functions of the SLC26 family of proteins have arisen from modifications of a common ancestral ion transport mechanism. Structural information often facilitates efforts to identify regions in proteins responsible for the expressions of their diverse physiological functions. Since the structure of the TM region has only been solved for a bacterial SLC26 protein, SLC26Dg (Fig. 1A) (7), we modeled the TM structures of pendrin and prestin based on the solved structure of SLC26Dg (Figs. 1B and 1C). Two prominent extracellular loops, which are referred to as loop-1 (L1) and loop-2 (L2) in this study, are present in pendrin and prestin, but not in the bacterial SLC26 protein (shown in magenta in Figs. 1B and 1C). Since many disease-associated pendrin mutations have been identified in the L1 (F1611, V1631, V163L, S166N, and T178P) and L2 (V250A, S252P, I253V, L257P, D266N) regions (11-19), these extracellular loops may play significant roles in defining the physiological functions of pendrin (HCO3-/I-/Cl- exchanger) and prestin (voltage-operated motor).

The extracellular loops modulate the voltage-operating point of prestin

In order to examine the contributions of L1 and L2 to the function of prestin, we generated several prestin-based constructs whose L1 and L2 are replaced with those of pendrin individually (A5-L1 and A5-L2) or in combination (A5-L1/L2) (Figs. 1 and 2), and measured NLC in HEK293T cell lines expressing these constructs (Fig. 2). Replacement of L1 did not abrogate NLC but resulted in significant hyperpolarization of the voltage-operating points (Vpk) (Figs. 2B and 2E). Likewise, replacement of L2 resulted in significant Vpk hyperpolarization but to a much greater degree compared to L1 replacement (Figs. 2C and 2E). No further hyperpolarization was observed upon double (L1/L2) replacement (Figs. 2D and 2E). The voltage sensitivity (α) was not significantly affected by any of these loop-replacements (one-way ANOVA, P = 0.14) (Fig. 2F). These results suggest that the extracellular loops of prestin modulate its operating voltage range without affecting its voltage sensitivity.

Pendrin possesses voltage-sensing ability

The voltage-driven motor function of prestin is thought to be unique among the members of the SLC26 family, however, the veracity of this presumption has not been tested rigorously. Given
the high degree of similarity in amino-acid sequences among the SLC26 family members, it is possible that many, if not most, other SLC26 proteins also retain a voltage-sensing mechanism but not readily detectable within experimentally measurable voltage range (typically ±150 mV). We noticed that some HEK293T cells expressing the wild-type pendrin construct tend to exhibit gradual increase in cell membrane capacitance below ~0 mV, hinting at the presence of large NLCs (Fig. 3A). We speculated that pendrin also retains voltage-sensing ability but its $V_{pk}$ is extremely hyperpolarized and thus not easily detected. To test this possibility, we repeated capacitance measurement for wild-type pendrin construct in the presence of methyl-β-cyclodextrin (MβCD), which is known to depolarize $V_{pk}$ of prestin by removing cholesterol from the cell membrane (20). As shown in Fig. 3B, NLCs of wild-type pendrin became quite evident upon application of MβCD due to its anticipated depolarizing effect, which brought otherwise barely detectable NLC of pendrin into experimentally measurable voltage range. We also performed NLC measurement in Sf9 cells (Fig. S1). The low membrane cholesterol level in this cell line (21) allowed detection of large NLC of pendrin without the use of MβCD.

The importance of the extracellular loops of pendrin for membrane targeting and modulation of the voltage-operating point

We examined the contributions of L1 and L2 to the voltage-sensing function of pendrin as we did for prestin. To this end, we generated several pendrin-based constructs whose L1 and L2 are replaced with those of prestin individually (A4-L1 and A4-L2) or in combination (A4-L1/L2) (Figs. 1 and 3). We anticipated to find large NLCs for A4-L1 since the L1 region contains the “14-aa” segment that was reported to confer large NLC and electromotility on pendrin (22). However, the effect of L1 replacement was ambiguous (Fig. 3C), as NLCs of A4-L1 (and wild-type pendrin, wt-A4) were only partially measurable to confidently estimate the NLC parameters. Qualitatively, the L1 replacement did not seem to significantly affect $V_{pk}$ of pendrin (Figs. 3A and 3C). We also generated a construct in which only the 14-aa segment used in the previous study (22) was replaced (termed “A4-L1(14aa)” in this study, Fig. 1D). Contrary to anticipation, however, we could not detect NLC for this construct (Fig. 3D). The reason for the discrepancy is unclear. At the least, the absence of NLC in A4-L1(14aa) should not be solely ascribed to impaired membrane targeting of the construct because the HCO3-/Cl- antiport activity was detected for A4-L1(14aa) (Fig. 4A). Although the transport activity of A4-L1(14aa) was smaller than wt-A4 ($P = 0.0016$) (Fig. 4A), it was comparable to that of A4-sL2 ($P > 0.999$, Fig. 4A) that exhibited a large NLC (Fig. 3F, see below).

Replacement of L2 (A4-L2) or both L1/L2 (A4-L1/L2) resulted in loss of NLC (Fig. 3E) and HCO3-/Cl- antiport activity (Fig. 4A). This is very likely due to the lack of membrane targeting, as they are predominantly cytosolic and are qualitatively very different from wt-A4 (Fig. 4B). In order to circumvent this membrane targeting issue, we generated additional pendrin constructs with a shorter L2 (“sL2”, Fig. 1E) segment replaced (termed “A4-sL2”, “A4-L1/sL2”, and “A4-L1(14-aa)/sL2” Fig. 3). The replacement of the shorter L2 segment (A4-sL2, Fig 3F) resulted in the depolarization of $V_{pk}$ (as in MβCD-treated wt-A4) (Fig. 3F), indicating this segment similarly modulates voltage operating point in pendrin as in prestin. However, we did not detect any NLC in A4-L1/sL2 and A4-L1(14-aa)/sL2 (Fig. 3G). Since the HCO3-/Cl- antiport activity was not detected for A4-L1/sL2 and A4-L1(14-aa)/sL2, either (Fig. 4A), we examined the expression of A4-L1/sL2 and A4-L1(14-aa)/sL2 and found that they failed to target plasma membrane (Fig. 4B). These negative observations on A4-L1/sL2, A4-L1(14-aa)/sL2, and A4-L1(14-aa)/sL2 imply the importance of pendrin’s extracellular loops for maintaining its structural integrity and/or membrane targeting.

The role of the charged residues in loop-2 in establishing $V_{pk}$

The L2-swapping between prestin and pendrin resulted in hyperpolarization for prestin and depolarization for pendrin (Figs. 2 and 3). This inverse effect of the L2-swapping can be explained by the electrostatic property of the L2 region that is presumably expected to be exposed to the extracellular surface of the membrane. For example, the presence of acidic residues would decrease the surface charge density ($\sigma_o$), which should result in concomitant drops
Pendrin retains voltage-sensing ability of the membrane surface potential ($\Phi_0$) (Fig. 5A). The opposite (depolarization of $\Phi_0$) would be expected if basic residues were present in L$_2$ (Fig. 5B). If the inner surface potential ($\Phi_i$) remained unchanged, hyperpolarization of $\Phi_0$ should result in hyperpolarization of $V_{pk}$, and vice versa. This interpretation is compatible with the fact that the net calculated charges of L$_2$ are -1 and +4 for pendrin and prestin, respectively (Fig. 1E).

E259 is the sole charged residue present in sL$_2$ of pendrin, whereas none is present in that of prestin (Fig. 1E). In order to test whether the significant $V_{pk}$ depolarization found in A4-sL$_2$ (Fig. 3F) can be attributed to neutralization of the negative charge pertaining to E259, additional pendrin constructs harboring E259Q, E259R, or E259K mutation were generated, and their NLCs measured. NLCs became clearly measurable upon neutralization of E259 (E259Q) (Figs. 5C and 5J), which resemble those found in A4-sL$_2$ (Fig. 3F). Polarity reversal of E259 (E259R and E259K) also made NLC of pendrin clearly detectable within the measurable voltage range due to $V_{pk}$ depolarization (Figs. 5D, 5E, and 5J). We also examined the effect of the negative charge pertaining to D266, which is not located within the sL$_2$ region but located in L$_2$ (Figs. 1E). We found that neutralization of this negative charge (D266N) also depolarizes $V_{pk}$, and makes large NLC of pendrin readily detectable (Figs. 5F and 5J). Simultaneous introduction of charge-affecting mutations to E259 and D266 (A4-E259Q/D266N, A4-E259R/D266N, and A4-E259K/D266N) also depolarized $V_{pk}$, but to greater extents (Figs. 5G, 5H, 5I, and 5J). These results suggest that the effects of these charge manipulations are additive. In fact, we found a statistically significant linear correlation ($r = 0.85$, $P = 0.008$) between the calculated net charge in L$_2$ vs. $V_{pk}$ (Fig. 5K). The $\alpha$ value and its variation were similar among the pendrin constructs (one-way ANOVA, $P = 0.39$) (Fig. 5L), which argued against the possibility that the $V_{pk}$ values were significantly over- or underestimated. These observations are uniformly explained by depolarization of $\Phi_0$ as a result of reduction of the negative surface charge present in sL$_2$ or L$_2$ (Fig. 5A and 5B).

Charge manipulations of D271 (D271N, D271R, D271K), which is located in the transmembrane helix that immediately follows L$_2$ (Fig. 1), significantly impaired the targeting of pendrin to the cell membrane (Fig. S2A). Consistently, these mutants showed little or no NLC and transport activity (Fig. S2B and S2E), implying the importance of D271 for maintaining the structural integrity of the transmembrane region.

The relationship between the voltage-sensing ability of pendrin and its anion transport function
The voltage-driven motor function of prestin has been presumed to stem from a common anion transport mechanism that is likely shared among the members of the SLC26 family (23,24). In order to define the molecular mechanism underlying electromotility, it is important to examine whether the anion transport activity of prestin is affected by voltage. Unfortunately, prestin is not suitable for pursuing this possibility because we could not detect HCO$_3$/Cl$^-$ antipor activity for prestin (wt-A5) or any of our prestin-based constructs. We therefore examined the NLC-anion transport relationship in pendrin since we identified large NLC in pendrin (Fig. 3). The resting membrane potentials of HEK293T cells expressing wild-type pendrin (mean ± s.d.) were $-15.0 \pm 5.8$ mV (n = 12) and $-12.3 \pm 4.4$ mV (n = 16) in the presence and absence of M$\beta$CD, respectively, with no statistically significant difference among the groups (P = 0.17). If the voltage-sensing mechanism were intimately related to the anion transport mechanism, augmentation of the anion transport activity would be expected upon application of M$\beta$CD due to significant shift of $V_{pk}$ towards the resting membrane potential of the pendrin-expressing cells (Figs. 3A and 3B). However, we did not find statistically significant change in the anion transport activity upon application of M$\beta$CD ($P > 0.999$, Fig. 4A), suggesting independence of the transport and voltage-sensing mechanisms.

NLC1 and NLC2 segments of pendrin and prestin are not mutually interchangeable
Previous efforts to identify a segment(s) in the prestin molecule that is essential for the expression of electromotility have been inconsistent. One study (25) identified such segments termed “NLC1” and “NLC2” in the region forming the
Pendrin retains voltage-sensing ability whereas other studies identified a short segment (termed “14-aa” in the present study) located in L1 (22, 26). These results are apparently irreconcilable since the short 14-aa segment is not a part of the large NLC1/NLC2 segments. Since we could not confirm the importance of the 14-aa short segment for the expression of NLC (Fig. 3D), we also generated eight chimeric constructs in which the NLC1 and NLC2 segments were swapped between pendrin and prestin (A4A5Ch1 - A4A5Ch8) (Fig. 6A), which are similar to those generated by Schaechinger et al. using electromotile and nonelectromotile prestin orthologues (25). Unfortunately, none of our pendrin/prestin chimeras showed NLC or HCO3-/Cl- antiport activity (Figs. S2C, S2D, and S2E) probably due to the lack of membrane targeting (Fig. 6B), suggesting a difference in the intramolecular interactions within the TM domain between pendrin and prestin.

**DISCUSSION**

The electromotile function of prestin (SLC26A5) is thought to be unique among the members of the SLC26 family. Although not all prestin orthologues exhibit motor activity, they all show NLC (27-30). NLC indicates the presence of a rapid voltage-sensing mechanism, and it is directly coupled to concurrent motor activity in electromotile prestin orthologues (31-34). In the present study, we show that pendrin (SLC26A4) also exhibits NLC but with extremely hyperpolarized Vpk. Given the high degree of similarity in amino-acid sequences among the SLC26 proteins, the presence of NLC in other members in the family would not be surprising, but this possibility has not been thoroughly examined heretofore. A previous study also reported NLC parameters for pendrin-expressing cells (22). However, the magnitude of the NLCs were much smaller than those observed in the present study despite their apparent Vpk (~ -40 mV) located within an easily measurable voltage range (22). In addition, the small NLCs found in the previous study were qualitatively very different from those reported in this study, as the Vpk values were depolarized, but not hyperpolarized, with respect to Vpk of prestin (22). We claim that the NLCs detected in the present study for the pendrin-expressing cells are indeed mediated by pendrin because we found that the Vpk of the NLCs responds to cholesterol depletion by MβCD (Fig. 3) in the same way as previously demonstrated for prestin (20), and that swapping of L2 that contains opposite electrostatic property between pendrin (net negative) and prestin (net positive) results in Vpk shifts towards mutually opposing directions for pendrin (depolarization, Fig. 3) and prestin (hyperpolarization, Fig. 2). Successful identification of large NLCs in pendrin-expressing SF9 cells without the use of any genetic or chemical manipulations further authenticates our claim (Fig. S1).

There is controversy over the identity of the voltage-sensing charge (6, 35-38). An early study proposed an incomplete transport model to explain the voltage-sensing mechanism of prestin where intracellular Cl- or HCO3- is used as an extrinsic voltage sensor (35). The basic notion of this model does not disagree with the fact that the HCO3-/Cl- antiport activity of prestin is very low (39) or virtually undetectable. However, later studies found that electromotility can be conferred on nonelectromotile SLC26 proteins without diminishing or abrogating the transport activities (25, 26). Our finding that pendrin exhibits both significant HCO3-/Cl- antiport activity and NLC (Figs. 3 and 4) underscores that the anion transport function and voltage-sensing ability are not mutually exclusive. The identification of large NLC in pendrin provided a fortuitous opportunity to examine the functional relationship between voltage-sensing and anion transport mechanisms, which could not be pursued in prestin due to its extremely small anion transport activity to be compared with NLC. We found that the HCO3-/Cl- antiport activity of pendrin is not affected by significant depolarizing shift of NLC towards the resting membrane potentials of pendrin-expressing cells upon MβCD application (Figs. 3 and 4). This observation suggests that voltage-sensing and anion transport mechanisms may not be intimately related, which agrees with a previous observation suggesting separation of voltage-sensing and anion transport functions for prestin (38).

It is possible that most, if not all, members of the SLC26 family retain voltage-sensing ability. It is important to note that the absence of measurable NLC does not necessarily mean inability of an SLC26 protein to sense voltage. Since experimentally measurable range of
membrane potential is limited, NLC with extremely hyper/depolarized $V_{pk}$ is difficult to be identified. In the present study, we demonstrate that this is, in fact, the case for pendrin (Fig. 3A). Fast voltage-sensing kinetics is another important factor for a successful NLC recording since detections of capacitative currents is the basis of NLC measurement. If voltage-sensing charge movement is very slow, it would not manifest as NLC. For example, NLC of zebrafish prestin shows significant stimulus voltage-frequency-dependence, and it becomes unmeasurable at high stimulus frequencies due to its relatively slow voltage-sensing kinetics (27). It is conceivable that the voltage-sensing kinetics of some SLC26 members may be too slow to exhibit its voltage-sensing ability as NLC. Collectively, absence or abrogation of NLC by a genetic or pharmacological manipulation needs to be concluded carefully.

For physiological electromotile function of prestin orthologues, it is important to set their $V_{pk}$ within the physiological $V_m$ range. Our study demonstrate that the charged residues present in $L_2$ greatly contribute to set distinct $V_{pk}$ for prestin and pendrin (Figs. 2, 3 and 5), and that the directions of $V_{pk}$ shift induced by charge-affecting mutations in $L_2$ can be simply explained by changes in the membrane surface charge density ($\sigma_o$) that concomitantly affects membrane surface electrical potential ($\Phi_o$) (Figs. 5A and 5B). Furthermore, all previously reported $V_{pk}$ shifts of prestin that were induced by charge-affecting mutations introduced to $L_2$, i.e., T234K (depolarization) (40), K233Q/K235Q/R236Q (hyperpolarization) (35), S238D (hyperpolarization) (41), and K255Q (hyperpolarization) (38), can be explained by their anticipated effects on the membrane surface potential. On the contrary, $L_1$ does not seem to contribute to confer distinct $V_{pk}$ on pendrin (Fig. 3C). Although significant $V_{pk}$ hyperpolarization was observed in prestin upon $L_1$ swapping (Fig. 2B), no additive effect on $V_{pk}$ was found upon $L_1/L_2$ double swapping (Fig. 2D). In any case, the hyperpolarizing $V_{pk}$ shift of prestin upon $L_1$ swapping (Fig. 2B) cannot be explained by the electrostatic property of $L_1$ because the net charge of $L_1$ is almost the same between prestin and pendrin (Fig. 1D). Also, hyperpolarizing $V_{pk}$ shifts of prestin induced by D154N and D155N mutations (35) cannot be explained by an $L_1$-associated surface charge effect. These irregular and unpredictable consequences of $L_1$-swapping may imply a dynamic role of the $L_1$ region in prestin.

Both single ($L_1$ or $L_2$) and double ($L_1$ and $L_2$) loop swapping of the extracellular loops do not deprive prestin of its function (Fig. 2). Pendrin also tolerates single ($L_1$ or $L_2$) swapping, but neither single $L_2$- nor double $L_1/L_2$-swapped pendrin constructs (A4-L2 and A4-L1/L2) showed NLC or HCO$_3^$/Cl$^-$ antiport activity, likely due to impaired membrane targeting (Fig. 4B). We eluded the membrane targeting issue for A4-L2 by shortening the swapped $L_2$ region (sL2) (Fig. 3F). However, double loop-swapped pendrin constructs with sL2 (A4-L2/sL2 and A4-L1L2(14aa)/sL2) still fail to target the membrane (Fig. 4B). These observations, together with our negative observation on the eight pendrin/prestin chimeras (Fig. 6), suggest a difference in the intramolecular interactions between pendrin and prestin, or may imply a difference in the TM domain architecture between pendrin and prestin.

It is noteworthy that the voltage sensitivity of pendrin, which is reflected in its $\alpha$ value, is 2-3 times smaller than that of prestin (Figs. 2F and 5L), and that the $\alpha$ values of nonelectromotile prestin orthologues are also smaller than those of electromotile ones (27,28). Since $\alpha$ values would indicate the magnitude and extent of voltage-induced conformational change (see EXPERIMENTAL PROCEDURES), it is interesting to examine if and how the magnitudes of $\alpha$ correlates with the expression of electromotility. Although electrophysiological characterization has already been conducted for various prestin orthologues (27-30), similar efforts have not been expanded to the other members in the SLC26 family. Thorough comparative studies across the SLC26 family would facilitate efforts in defining the molecular mechanisms underlying the uniqueness and generality of electrophysiological characteristics among the SLC26 family members.

**EXPERIMENTAL PROCEDURES**

**Generation of stable cell lines that express various SLC26 protein constructs**

The pendrin (UniProt ID: O43511)- and prestin (UniProt ID: Q9JKQ2)-based constructs were generated by multi-step PCR (A4-L1, A4-L2, A4-
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L1/L2, A5-L1, A5-L2, and A5-L1/L2) (42), site-directed mutagenesis (A4-E259Q, A4-E259R, A4-E259K, A4-D266N, A4-E259Q/D266N, A4-E259R/D266N, A4-E259K/D266N), and de novo DNA synthesis combined with template vector PCR (A4-sL2, A4-L1/sL2, A4-L1(14aa), A4-L1(14aa)/sL2, A4A5Ch1-A4A5Ch8). The complete DNA sequences of all these constructs, along with the PCR primers and synthesized double-stranded DNA information, are provided in a supplemental file. These constructs with C-terminally attached ECFP (for prestin-based constructs) or mTurquoise2 (for pendrin-based constructs) were cloned into a pSBetet-Pur vector (addgene, Cambridge, MA) (43). The functions of pendrin and prestin are not abrogated by a C-terminally fused fluorescent protein (32,44). Stable cell lines that express these recombinant protein constructs were established in HEK293T cells as described previously (43). Expressions of the pendrin/prestin constructs were induced by application of doxycycline (1µg/ml) to the cell culture media 1-3 days prior to experiments.

**Protein structure modeling**

The structural models of the transmembrane regions of pendrin and prestin were generated by Phyre2 (45) based on the SLC26Dg structure (PDB ID: 5DA0) (7) using the partial amino acid sequences of pendrin (Pro76 – Arg512) and prestin (Pro72 – Arg502). The models were rendered by Pymol (https://pymol.org) (Fig. 1) or CueMol software (http://www.cuemol.org) (Fig. 6).

**Electrophysiology**

Whole-cell recordings were performed at room temperature (RT) using the Axopatch 200A/B amplifier (Molecular Devices, Sunnyvale, CA). Recording pipettes were pulled from borosilicate glass to achieve initial bath resistances averaging 3-4 MΩ. Whole-cell nonlinear capacitance (NLC) recordings were performed using a 0 mV holding potential and a sinusoidal voltage stimulus (2.5-Hz, 120-150 mV amplitude) superimposed with two higher frequency stimuli (390.6 and 781.2 Hz, 10 mV amplitude). Recording pipettes were filled with an intracellular solution containing (mM): 140 CsCl, 2 MgCl2, 10 EGTA, and 10 HEPES (pH 7.3 adjusted at RT). Cells were bathed in an extracellular solution containing (mM): 120 NaCl, 20 TEA-Cl, 2 CoCl2, 2 MgCl2, 10 HEPES (pH 7.3 adjusted at RT). Osmolarity was adjusted to 310 mOsm/l with glucose (34,46). Intracellular pressure was kept at 0 mmHg and current data were collected by jClamp (SciSoft Company, New Haven, CT) using a fast Fourier transform-based admittance analysis to determine NLC (47). The resting cell membrane potentials were determined in HBSS (14025, ThermoFisher Scientific, Waltham, MA) using recording pipettes filled with an intracellular solution containing (mM): 140 KCl, 2 MgCl2, 10 EGTA, and 10 HEPES (pH 7.3 adjusted at RT).

**NLC data analysis**

Voltage-dependent cell membrane electric capacitance data were analyzed using the following two-state Boltzmann equation:

\[ C_m = \frac{\alpha Q_{max} \exp[\alpha(V_m - V_{pk})]}{(1 + \exp[\alpha(V_m - V_{pk})])^2} + C_{lin} \]

where \( \alpha \) is the slope factor of the voltage-dependence of charge transfer, \( Q_{max} \) is the maximum charge transfer, \( V_m \) is the membrane potential, \( V_{pk} \) is the voltage at which the maximum charge movement is attained, and \( C_{lin} \) is the linear capacitance (34,46,48,49). In this two-state Boltzmann model, \( \alpha \) is defined as \( z_{app} e / k_b T \), where \( z_{app} \) is the apparent valence of charge movement, \( e \) is electron charge, \( k_b \) is the Boltzmann constant, and \( T \) is absolute temperature. The magnitude of \( z_{app} \) reflects the valence (\( \delta \)) along the direction of the electric field of a voltage-sensing charge.

**Anion transport assay**

A ratiometric fluorescent pH indicator, SNARF-5F (S23923, ThermoFisher Scientific), was loaded into cells in a high chloride buffer containing (mM): 140 NaCl, 4.5 KCl, 1 MgCl2, 2.5 CaCl2, 20 HEPES (pH 7.4 adjusted at RT, 320 mOsm/l) for 30 min in the presence of 5% CO2 at RT. The cells were washed with the high chloride buffer once, and resuspended in 100 µL of the high chloride buffer. Portions of the cell suspensions (50 µL) were transferred to wells in a 96-well plate (~1.5x10^5 cells/well). HCO3/C1 antip port assay was initiated by an automated injection of 200 µL of a low chloride buffer containing (mM): 125 Na-glucconate, 5 K-glucconate, 1 MgCl2, 1 CaCl2, 20 HEPES, 25 NaHCO3 (pH 7.4 at RT under 5% CO2) in Synergy2 plate reader (BioTek, Winooski,
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The fluorescence intensity of SNARF-5F was measured in a time dependent manner. The excitation and emission filters used were 504/12 (Ex) (Semrock, Rochester, NY), 572/28 (F1) (Semrock), and 709/167 (F2) (Semrock), respectively. The fluorescence ratio, F2/F1, was converted into the intracellular H+ concentration using a pH calibration curve. The HCO3-/Cl- antiport activity [s^-1] was determined from exponential curve fitting.

**Confocal imaging**

The stable cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, high glucose, GlutaMAX, pyruvate, Thermo Fisher Scientific) with 10% FBS (French origin, BioWest, Riverside, MO) at 37 °C (5% CO2) in glass-bottom dishes (D11130H, Matsunami Glass, Kishiwada, Japan). When cell confluency reached 20-30%, expressions of the pendrin/prestin constructs were induced by 1 µg/mL doxycycline hyclate (LKT Laboratories, ST. Paul, MN) included in the medium for 2–3 days. Fluorescent images of the cells were acquired using an LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63x Plan-Apochromat objective lens (NA 1.40 Oil) and a filter set (BP 395-440 exciter filter, FT460 dichroic mirror, and LP470 emission filter). The cells were illuminated with a 30 mW 405-nm diode laser (laser power: 2%) at RT, and the images were taken with ZEN software (Carl Zeiss). The pinhole size and pixel dwell time were set to be 1 airy unit (490 nm) and 1.27 µs, respectively. Four sequentially scanned images were averaged, and then processed with a despeckle algorithm and contrast/brightness adjustment using Fiji software (http://fiji.sc).

**Statistical analyses**

Statistical analyses were performed using Prism (GraphPad software). The Student’s t-test was used for comparisons between two groups. One-way analysis of variance (ANOVA) combined with the Tukey-Kramer test was used for multiple comparisons. P < 0.05 was considered statistically significant.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.
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FOOTNOTES
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The abbreviations used are: SLC26, the solute carrier 26; NLC, nonlinear capacitance; MβCD, methyl-β-cyclodextrin;
Figure 1. Structural models of pendrin and prestin. (A-C) The extracellular views of pendrin and prestin models generated based on the solved structure of SLC26Dg. The N- and C-terminal cytosolic regions are not shown. The core and the gate domains are shown in cyan and orange, respectively. The two prominent extracellular loops (L$_1$ and L$_2$), which are not present in the bacterial SLC26 structure, are highlighted in magenta. (D-E) Partial amino acid sequences of SLC26A4 (pendrin) and SLC26A5 (prestin) that contain the L$_1$ (D) and L$_2$ (E) regions. Characters shown in cyan and magenta indicate acidic and basic residues, respectively. L$_1$, L$_2$, 14aa, and sL$_2$ indicate the segments that were swapped between pendrin and prestin in this study.
Figure 2. NLC measurements for prestin and prestin-based constructs. (A-D) Schematic diagrams of wild-type prestin (wt-A5) (A) and prestin-based constructs (A5-L1 (B), A5-L2 (C), and A5-L1/L2 (D)), along with their representative NLCs, are shown. The parts of prestin and pendrin are indicated by magenta and dark blue, respectively, in the schematic diagrams. The magnitudes of NLCs ($C_m - C_{lin}$) are corrected for cell size ($C_{lin}$) because larger cells tend to express greater amounts of prestin in their cell membranes ($NLC_{sp} \equiv (C_m - C_{lin})/C_{lin}$). Different colors indicate individual recordings. A two-state Boltzmann model (see EXPERIMENTAL PROCEDURES) was used to interpret the NLC data (solid lines). (E) The $V_{pk}$ values (mean ± s.d.) are as follows: wt-A5 [-91 ± 24 mV, (n=9)]; A5-L1 [-132 ± 31 mV, (n=13)]; A5-L2 [-169 ± 26 mV, (n=9)]; A5-L1/L2 [-170 ± 24 mV, (n=9)]. The asterisks indicate the degree of statistical significance (**$P < 0.01$; ****$P < 0.0001$). (F) The $\alpha$ values (mean ± s.d.) are as follows: wt-A5 [0.031 ± 0.006 mV$^{-1}$, (n=9)]; A5-L1 [0.026 ± 0.007 mV$^{-1}$, (n=13)]; A5-L2 [0.025 ± 0.005 mV$^{-1}$, (n=9)]; A5-L1/L2 [0.026 ± 0.005 mV$^{-1}$, (n=9)].
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Figure 3. NLC measurements for pendrin and pendrin-based constructs. Representative NLC recordings for wt-A4 (A), wt-A4 + MβCD (B), A4-L1 (C), A4-L1(14aa) (D). A4-L2 and A4-L1/L2 (E), A4-sL2 (F), and A4-L1/sL2 and A4-L1(14aa)/sL2 (G). Schematic diagrams of these constructs are also shown above the NLC data. The parts of prestin and pendrin are indicated by magenta and dark blue, respectively. The magnitudes of NLCs are corrected for cell size as in Fig. 2. Different colors indicate individual recordings. A two-state Boltzmann model was used to interpret the NLC data (solid lines) for wt-A4 + MβCD (B) and A4-sL2 (F). The $V_{pk}$ and $\alpha$ values are summarized in Figs. 5J and 5L.
Figure 4. A summary of anion transport assay and qualitative assessment of subcellular localization for the pendrin-based constructs that did not show transport activity. (A) HCO$_3$/$\text{Cl}^-$ antiport activities of non-induced negative control (n=7), wt-A4 (n=6), wt-A4 + MβCD (n=3), A4-L$_1$ (n=4), A4-L$_1$(14aa) (n=4), A4-L$_2$ (n=4), A4-L$_1$/L$_2$ (n=4), A4-sL$_2$ (n=3), A4-L$_1$/sL$_2$ (n=3), and A4-L$_1$(14aa)/sL$_2$ (n=4). The error bars indicate the standard deviations. The transport activities of A4-L$_2$, A4-L$_1$/L$_2$, A4-L$_1$/sL$_2$, and A4-L$_1$(14aa)/sL$_2$ were indistinguishable from that of the non-induced negative control (determined by the Student’s t-test and indicated by “ns”). The results of the other constructs that showed significant transport activities (compared to the non-induced negative control) were further analyzed by one-way ANOVA and the Tukey-Kramer multiple comparison test to determine the adjusted $P$ values. Only selected $P$ values that are referred in the main text (under Results) are shown in the figure. (B) The subcellular localizations of the pendrin constructs were microscopically examined. A4-L$_2$, A4-L$_1$/L$_2$, A4-L$_1$/sL$_2$, and A4-L$_1$(14aa)/sL$_2$, which did not show significant transport activity (A), exhibited predominantly cytosolic localizations, indicating severely impaired membrane targeting. A result for wt-A4 is also included as positive control. Scale bars, 10 µm.
Figure 5. The effects of the charged residues in L2 on Vpk. (A and B) Schematic representations of the cell membranes (yellow) and surrounding electrolyte solutions. \( \sigma_o \) and \( \sigma_i \) are charge densities at the outer and inner surfaces of the cell membrane, respectively. \( \Phi_o \) and \( \Phi_i \) are electric potentials at the outer and inner surfaces of a cell membrane, respectively. (C-I) Representative NLC recordings for A4-E259Q (C), A4-E259R (D), A4-E259K (E), A4-D266N (F), A4-E259Q/D266N (G), A4-E259R/D266N (H), and A4-E259K/D266N (I). The magnitudes of NLCs are corrected for cell size as in Figs. 2 and 3. Different colors indicate individual recordings. A two-state Boltzmann model was used to interpret the NLC data (solid lines). (J) The Vpk values (mean ± s.d.) are as follows: wt-A4 + MbCD [-166 ± 26 mV, (n = 12)]; A4-sL2 [-173 ± 22 mV, (n = 9)]; A4-E259Q [-184 ± 24 mV, (n = 10)]; A4-E259R [-154 ± 19 mV, (n = 9)]; A4-E259K [-134 ± 20 mV, (n = 10)]; A4-D266N [-189 ± 20 mV, (n = 11)]; A4-E259Q/D266N [-157 ± 16 mV, (n = 11)]; A4-E259R/D266N [-132 ± 21 mV, (n = 13)]; A4-E259KR/D266N [-149 ± 20 mV, (n = 12)]. (K) Correlation between the net charge in L2 vs. Vpk. The means and the standard deviations of the Vpk data shown in panel J are plotted against the calculated net charge in L2 (at pH 7.3). (L) The \( \alpha \) values (mean ± s.d.) are as follows: wt-A4 + MbCD [0.012 ± 0.002 mV^{-1}, (n = 12)]; A4-sL2 [0.010 ± 0.001 mV^{-1}, (n = 9)]; A4-E259Q [0.010 ± 0.002 mV^{-1}, (n = 10)]; A4-E259R [0.011 ± 0.002 mV^{-1}, (n = 9)]; A4-E259K [0.010 ± 0.002 mV^{-1}, (n = 10)]; A4-D266N [0.011 ± 0.002 mV^{-1}, (n = 11)]; A4-E259Q/D266N [0.010 ± 0.001 mV^{-1}, (n = 11)]; A4-E259R/D266N [0.011 ± 0.001 mV^{-1}, (n = 13)]; A4-E259K/D266N [0.012 ± 0.002 mV^{-1}, (n = 12)].
Figure 6. Eight NLC1/NLC2-focused pendrin/prestin chimeras used in this study. (A) Both schematic diagram and tertiary structural models (lateral and extracellular views) are shown for prestin-based...
Pendrin retains voltage-sensing ability (A4A5Ch1, A4A5Ch2, A4A5Ch3, and A4A5Ch4) and pendrin-based (A4A5Ch5, A4A5Ch6, A4A5Ch7, and A4A5Ch8) chimeras. The parts of prestin and pendrin are indicated by magenta and dark blue, respectively. The amino acids and their residue numbers at the boundaries of A4 and A5 are also provided. The C-termini of all the constructs were tagged with ECFP (for prestin-based constructs, A4A5Ch1-A4A5Ch4) or mTurquoise2 (for pendrin-based constructs, A4A5Ch5-A4A5Ch8). (B) Qualitative assessments of the subcellular localizations of the eight chimeras (A4A5Ch1-8) heterologously expressed in HEK293T cells. Results for wt-A5 and wt-A4 are also included as positive controls. The scale bars indicate 10 µm. All the eight chimeras show predominantly cytosolic localization, indicating severely impaired membrane targeting.
The extracellular loop of pendrin and prestin modulates their voltage-sensing property
Makoto F. Kuwabara, Koichiro Wasano, Satoe Takahashi, Justin Bodner, Tomotaka Komori, Sotaro Uemura, Jing Zheng, Tomohiro Shima and Kazuaki Homma

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