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The Molecular Basis of Outer Hair Cell Electromotility is Defined by Prestin's Conformational Cycle

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Abstract: The voltage-dependent motor protein Prestin (SLC26A5) is responsible for the electromotive behavior of outer hair cells (OHCs) and underlies the cochlear amplifier ¹. Knock out or impairment of Prestin causes severe hearing loss ²⁻⁵. Despite Prestin's key physiological role in hearing, the mechanism by which mammalian Prestin senses voltage and transduces it into cellular-scale movements (electromotility) is poorly understood. Here, we determined the structure of dolphin Prestin in six distinct states using single particle cryo-electron microscopy. Our structural and functional data suggest that Prestin adopts a unique and complex set of states, tunable by the identity of bound anions (Cl⁻ or SO₄⁼). Salicylate, a drug that can cause reversible hearing loss, competes for the anion-binding site of Prestin, inhibits its function by immobilizing locking in a novel conformation. This suggests that the anion together with its coordinating fixed charges act as a dynamic voltage sensor. Analysis of all anion-dependent conformations reveals how structural rearrangements in the voltage sensor are coupled to conformational transitions at the protein-membrane interface, suggesting a novel mechanism of area expansion. Visualization of Prestin's electromotility cycle distinguishes Prestin from closely related SLC26 anion transporters, highlighting the basis for evolutionary specialization of the mammalian cochlear amplifier at high resolution.

Main

Mammals have evolved a highly sophisticated sense of hearing⁶ characterized by extraordinary sensitivity and the ability to process high-frequency sounds^{7,8}. This evolutionary outcome is the result of a mechanism of amplification that relies on the specialized electromotility of the mammalian outer hair cells (OHCs)⁹⁻¹¹. The cochlear amplifier depends on the voltage-dependent longitudinal contractions or elongations of OHCs triggered by the concerted action of millions of fast “motor” proteins in the basolateral membrane¹². This molecular motor was identified as Prestin (SLC26A5), a piezoelectric member of the SLC26 family of anion transporters¹. Importantly, Prestin’s absence or dysfunctions are associated with non-syndromic hearing loss in mammals²⁻⁴. In spite of the intense attention to Prestin since its identification as the OHC motor¹, fundamental questions remain unanswered, including its oligomeric state¹³⁻¹⁶, underlying voltage sensing mechanisms, the molecular basis of electromotility, and the evolutionary relationship between Prestin and other, non-electromotive yet closely-related SLC26 family members¹⁷.

We have used single particle cryo-electron microscopy (cryo-EM) to determine the structure of Prestin under various ionic conditions and in complex with the reversible inhibitor salicylate. These structures, together with site-directed mutagenesis, functional data and electrostatic calculations show that Prestin adopts a defined set of states as part of its electromotility cycle. These states point to novel mechanisms of voltage-dependent area changes, highlight the evolutionary differences with SLC26 transporters, and constrain explicit models that help explain Prestin’s behavior as a piezoelectric motor.

Cryo-EM Structure of the SLC26A5 homodimer in Cl⁻

An initial screening by fluorescence-detected size-exclusion chromatography (FSEC) led to the identification of a Prestin candidate from the bottlenose dolphin (*Tursiops truncatus*). HEK293 cells expressing dolphin Prestin display the characteristic bell shape NLC with a $V_{1/2}$ of -61 ± 0.5 mV (mean $\pm$ SEM, n=20, Fig. 1a) and similar voltage sensitivity ($1/\alpha = 37 \pm 3$ mV, mean $\pm$ SEM, n=20) as those reported for other mammalian systems. Importantly, HEK293 cells overexpressing Prestin at high levels undergo unusually large voltage-driven cell movements under whole cell patch clamp configuration, consistent with robust levels of electromotility (Fig. 1b; Extended Data Fig. 1a; Movie S1). Expressed in baculovirus-infected mammalian cells, dolphin Prestin is characterized by a main gel filtration peak around ~150 kDa (predicted molecular mass of ~75 kDa), suggesting a stable dimer in solution (Extended Data Fig. 1b,c). We report no evidence of larger oligomeric assemblies.

We determined the structure of full-length dolphin Prestin at 3.3 Å using single particle cryo-EM, with Cl⁻ as the main anion (Fig. 1c, d; Extended Data Fig. 1d). It shows Prestin is a symmetric homodimer (C2) with 14 α -helical transmembrane (TM) segments (Extended Data Fig. 2), divided into two overlapping domains: a core domain (TM segments 5-7 and 12-14) and a gate domain (TM segments 1-4 and 8-11) as part of the individual subunit. The TM regions are domain swapped with the STAS containing -N and -C termini cytoplasmic domains (Fig. 1d). This putative Cl⁻-bound conformation is structurally closest to that of the intermediate state in SLC26A9 (PDB:6RTF)¹⁸ (Extended Data Fig. 3), where a large cavity is observed accessible to the intracellular face of the molecule between gate and core modules. We see no explicit density associated with a bound Cl⁻, as is the case with other SCL26 structures^{18,19}.

SO₄²⁻ binding uncovers novel Prestin conformations from sensor Down (Expanded) to sensor Up (Compact) states

Replacing Cl⁻ with SO₄²⁻ as the main anion (10 mM Cl⁻ vs. ~ 130 mM Na₂SO₄²⁻) reduces dolphin Prestin’s NLC by ~ 75 % and shifts it ~80 mV toward positive potentials (Fig. 2a). This is in agreement with previous studies of Prestin from other species²⁰⁻²⁵. Therefore, at zero mV almost

half of its total charge has moved across the electric field and SO_4^{2-} -Prestin would display maximal conformational heterogeneity. Indeed, the cryo-EM structural determination of SO_4^{2-} -Prestin revealed three distinct new states: two conformations with solvent access to the anion binding pocket (Down I and II) and an Intermediate conformation preceding the Up state (Fig. 2b-d, Extended Data Fig. 4). When compared with Cl^- -Prestin (Up), the major differences among these states were quantitatively described based on two measurements between Core and Gate domains: z , the position of residue R399 (TM10, Core domain) relative to V499 in TM14 in the gate domain²⁵; and d , the gap that separates the TM3-TM10 helical dipole (Fig2.b).

When linked as part of a trajectory of conformational rearrangements, these states comprise the overall working cycle of Prestin, from Down to Up conformations. In the Down states the core domain is positioned towards the intracellular face of the membrane (z between 8-10 Å). While in the Up state, the core domain moves ~ 6 Å towards the extracellular face of the membrane (Fig. 2b,c). The movement (Fig. 2c, Movies S2) is reminiscent of a partial “elevator” rearrangement seen in other SLC transporters where the uppermost conformation in Prestin would go beyond SLC26 transporters in an occluded (Intermediate) state¹⁸. Moreover, the core domain movements associated with the substitution of Cl^- by SO_4^{2-} also trigger a unique widening of the anion binding pocket (parameter d). Molecular dynamics simulations confirm that these structural changes lead to substantial changes in water penetration, where the anion binding pocket is fully water accessible in states Down I and II but water accessibility is sharply reduced in the Intermediate and Up states (Fig. 2d). Even when the prestin voltage sensor is in a maximally Up conformation, the anion binding site is still not exposed to the extracellular side, preventing release of anions to the extracellular space. This inability to complete a canonical transport cycle rationalizes why Prestin is unable to efficiently transport anions, unlike other members of the SLC26 family.

As proposed, force transmission through an area motor model²⁶⁻²⁸ requires Prestin to populate at least two distinct conformations characterized by different intra-membrane cross sectional areas. Indeed, the movement of the binding pocket from Down to Up is accompanied by an eccentric contraction of the Prestin’s inter-membrane cross-sectional area, where the (Cl^- -bound) Up conformation corresponds to a Compact state and the (SO_4^{2-} -bound) Down conformations represent Expanded states. These two sets of conformations differ in cross sectional area by ~10% (Fig. 2b, Extended Data Fig. 5), pointing to a mechanism where Prestin might exert lateral forces that directly influence the physical state of the bilayer. Moreover, this feature seems to be unique to the motor function when compared with the Cl^- transporter SLC26A9¹⁸ (Extended Data Fig. 5).

Salicylate traps Prestin in an Intermediate state by occluding the anion-binding pocket.

The amphiphilic drug Salicylate is known to cause tinnitus^{29,30}, abolishing the NLC and electromotility in mammalian OHCs^{1,31,32}, while also inhibiting Cl^- transport in its non-mammalian homologs³³. Whole-cell patch clamp recordings show that 10 mM Na-salicylate flattens dolphin Prestin’s NLC curve across the physiological voltage range (Fig. 3a, Extended Data Fig. 6)^{1,31,32}. However, the molecular basis for Salicylate inhibitory effects remains unclear. We determined the structures of the Prestin-salicylate complex in the presence of either, Cl^- (360 mM) or SO_4^{2-} (120 mM) at 3.8 Å and 3.7 Å, respectively (Fig. 3b, Extended Data Fig. 7). In each case, 50 mM sodium salicylate was present.

Salicylate likely outcompetes any other bound anion at the binding pocket, and given that no anion can be resolved even under saturating Cl^- or SO_4^{2-} concentrations (Fig. 3b), this density helps confirm the overall nature of ion coordination at the anion-binding pocket. In both structures, the salicylate density snugly fits in the pocket formed by residues Q97, F101, F137, L397, S398 and R399 (Fig. 3c). The majority of these residues are conserved across SLC transporters and other Prestin homologs (Extended Data Fig. 6c). Comparing the anion binding site in the Up conformation with

that of the Cl⁻ + salicylate structure (Inhibited I), shows how the core domain TM10 moves ~ 3 Å downward, creating enough space to accommodate the larger salicylate (Fig. 3c). The salicylate-inhibited structures (Cl⁻ and SO₄²⁻) appear to converge into an Intermediate state that display common conformations at the anion-binding site (Fig. 3d, left). The major differences are observed at the TM6-TM7 helices, in the periphery of the gate domain, facing the lipid bilayer (Fig. 3d right). Based on these results, we suggest that salicylate inhibits Prestin's motor function by immobilizing the relative displacement of the core domain around the anion-binding site.

Prestin's voltage dependence is defined by the interplay between fixed charges and bound anions

A large body of experimental evidence points to the complex nature of the voltage sensor in Prestin^{21,23,31,34}, where intrinsic charges or dipoles would interact with bound anions to generate the NLC, ultimately driving electromotility. Indeed, neutralizing residue R399 fully eliminates the NLC (Extended data Fig.8a,e) in mammalian Prestin¹³ while neutralization of a series of fixed charges (K276, K359, K56 and K449) have a partial effect on unitary charge transfer^{35,36}. And yet, Prestin voltage dependence shows an acute dependence to the binding and occupancy of anions (Fig.2a, Extended data Fig.8b). The major effect of anions on Prestin's function is to shift Prestin's V_{1/2} along the voltage axis (Fig.2a). This simple experimental manipulation allows for a structure-based estimate of Prestin's electrostatic properties (Extended data Fig.8b-d), and by extension, a glimpse at the mechanism of voltage dependence. Due to the presence of a relatively large intracellular water-filled cavity, the electric field displays fairly limited focussing (in contrast to voltage sensing domains in ion channels and enzymes^{37,38}). Within this profile, it is clear that the anion-binding pocket is sensing only ~30-35% of the field. As a result, positive fields at the anion-binding pocket are only partially neutralized in the presence of a bound anion, regardless of their valence. In principle, this would explain the apparently paradox posed by the non-correlated effects of anion valence with the elementary charges estimated from NLCs^{21,23}. Voltage dependence can be estimated from the movement of the charged residues within a defined electric field³⁹. Gating charge calculations carried out based on the two extreme conformations (Down I to Up states) estimate that ~0.4 unitary charges move across the electric field (Extended data Fig.8d). These estimates are roughly consistent with earlier evaluations of charge movement in Prestin^{40,41}. Thus, in addition to the TM3-TM10 helical dipole, the positive charges located at the bilayer mid-plane (particularly R399), create an attracting field for anion binding and likely define Prestin's voltage sensor.

TM6 helix flexibility is an evolutionarily conserved feature of Prestin electromotility

In all our conformations, TM6-TM7 at the interface of Prestin with the lipid bilayer has been the most dynamic entity, as evidenced by diffused cryo-EM density in this region. Compared to a relatively straight helical configuration in the Up state, TM6 helix bends ~65° outwardly most noticeably in Inhibited II (Fig. 3d). Around this region, Prestin appears to have a strong influence in the shape of its surrounding micelle (Extended data Fig.5d). In the Intermediate and Up conformations, the micelle surrounding Prestin is shaped as an elongated oblate which thickens towards the longitudinal ends of the dimer (viewed extracellularly). In contrast, the micelle in the Inhibited II state is not only wider and thinner along the sides, but it displays a "notch" that induces a dramatic thinning of the micelle. Molecular dynamics simulations show that TM6 bending dramatically thinned the lipid bilayer by up to ~25% at the lipid-Prestin interface (Fig. 3b, Extended data Fig.5b,c).

We have identified a series of glycine residues that seem to endow TM6 with its unusual flexibility. While G265 appears to be highly conserved across all vertebrates⁴², a series of 3 additional Glycines appear only among mammals (G263, G274 and G275) (Fig.3e) and helps define the phylogenetic relations between *bona fide* mammalian electromotive OHCs with and those of other vertebrates.

Two of these residues, G274 and G275, are located precisely at the hinge that allows TM6 bending in the inhibited II state. Notably, the C terminal end of the TM6 is also capped by a cluster of charged residues (Fig. 3f) a fact that likely contributes to the membrane thinning. To assess the importance of the TM6 glycines in Prestin function, we systematically mutated them and the individual mutants were subsequently evaluated by patch clamp electrophysiology. Mutating G274 and G275 to Leucine and Valine (GG-LV) abolishes Prestin NLC (Fig. 3f), without changing the levels of expression. The potential role helical flexibility has been suggested previously⁴³. Aligned with these observations, electromotility and NLC are strongly influenced by the thickness of the bilayer^{44,45} as well as by membrane tension generated from surface area changes^{41,46,47}.

The structural rearrangements that causes change in the cross-sectional area between these states are not limited to TM6-TM7, as there are substantial conformational changes along other peripheral helices: TM5b (gate), and TM8 (core) (Extended data Fig.6a). Thus, the present data clearly points to a model where TM6 helical hinge-bending, the “electromotility elbow”, together with localized rearrangements on TM5, TM7 and TM8 underlies the putative transition between “compact” and “expanded” Prestin states (Fig.4). This is fully consistent with the process of OHC electromotility, where cell elongation coincides with an expanded Prestin state (hyperpolarization), while depolarization-driven contractions correlate with a compact Prestin state^{27,41,48-52}.

A putative molecular mechanism for OHC electromotility

Here, we have determined a complex set of interrelated conformations that define Prestin electromotility cycle at the molecular level (Fig 4a). Under physiological conditions, where Cl⁻ is the major intracellular anion, Prestin populates its most compact state with its voltage sensor in the Up conformation at 0 mV. Displacement of Cl⁻ by SO₄²⁻ shifts V_{1/2} ~100 mV in the depolarizing direction, which allowed us to sample both, expanded and intermediate conformations. Additional inhibited conformations (stabilized with bound salicylate) show expansion at TM6 in the membrane-protein interface. Based on these structures, we have been able to delineate the intricate sequence of conformational changes that take place in each Prestin monomer (Fig 4b), and by doing so, propose an explicit molecular mechanism of OHC electromotility. Transitions between the compact (Up) and Expanded (Down) conformations are accompanied by changes in the TM3-TM10 helical macrodipole (putatively affecting anion binding) and a displacement of the Core relative to the Gate domain. These movements appear to be transmitted allosterically to the helices in the gate domain (TM6-TM7) so that during the transitions from Down to Up conformation, the intra-helical spaces of the core domain contracts, leading to the reorientation of the TM6 electromotility elbow and TM1 helix.

Prestin is present in the lateral wall of OHCs at very high densities (~5,600-7000/μm²)¹² where they may interact cooperatively, directly, or through yet to be identified adaptor proteins. The efficiency of force transfer to the OHC lateral membrane depends on how individual motors are arranged in the plasma membrane, whether Prestin is vectorially or randomly oriented. Based on a model of the OHC lateral membrane with fully polarized Prestins along the longitudinal direction of the area change, the maximum expected change in cell length would be ~ 2.6 μm, or 5 % elongation relative to the initial length (See methods; Eq. (3)). This estimate is fully consistent with experimental values where OHC elongates/contracts within ~4% of length change^{48,52,53}. This estimate suggests that electromotility in OHCs is likely intrinsic to the changes in the physical state of the plasma membrane. However, a definitive understanding of the molecular and cellular basis of OHC electromotility will necessitate consideration of additional structural rearrangements and potentially other molecular actors (e.g. cytoplasmic elements).

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Data and materials availability: The atomic structure coordinates are deposited in the RCSB Protein Data Bank (PDB) under accession numbers XXXX XXXX, XXXX, XXXX, XXXX and XXXX; and the EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers YYYYY, YYYYY, YYYYY, YYYYY, YYYYY and YYYYY. All materials generated during the current study are available from the corresponding author under a materials transfer agreement with The

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Fig.1| Structure and function of dolphin SLC26A5 homodimer in Cl⁻. **a**, Normalized change in nonlinear capacitance (NLC= $C-C_{lin}$) and charge movement (right panel) of dolphin Prestin expressed in HEK cells and using patch clamp electrophysiology. Compared to mock (GFP only) transfected cells, dolphin Prestin has a bell shape change in capacitance with $V_{1/2}$ of -61 ± 0.5 mV ($n=20$, mean $\pm$ SEM). The NLC curve of Prestin (grey) vs transfected cells with GFP alone (blue, $n=8$) in whole-cell patches. Prestin's NLC follows a Boltzmann distribution (fit with solid lines). Cell movement is **b**, Electromotility of Hek293 cells transfected with dolphin Prestin in whole cell configuration ($n=7$). Cells were lifted off the surface to avoid any possible effect from cell-surface adhesion forces on the cellular movement. Upon changing the membrane potential (the amplitude were increased in each step), the overexpressing dolphin Prestin in HEK293 cells can visibly deform the cells. By tracking the change in the 2D projected area of the cell (red and yellow traces), the cell expands upon hyperpolarization. Cells transfected with GFP only does not show such response (blue solid line, $n=5$). Cell movement (right panel) has been normalized with the largest diameter of the cell, d_0 . **c**, Cryo-EM density map and the overall structure of the dolphin SLC26A5 homodimer at 3.3 $\text{\AA}$ nominal resolution when Cl⁻ is the main anion (See methods); the subunits

are colored in violet and green. **d**, Side and top views of Prestin structure colored according to the different domains, with the core domain in red, the gate domain in blue, and the N- and C-terminus and STAS domain in grey. UCSF ChimeraX was used for illustration.

Fig.2| Sulfate (SO₄²⁻) drives Prestin towards down and intermediate states at zero membrane potential. **a**, Normalized NLC and charge transfer measurement of transiently transfected HEK293 cells with dolphin Prestin with Cl⁻ and SO₄²⁻. Compared to Cl⁻, when SO₄²⁻ is the main internal and external anion, the NLC amplitude is attenuated and the V_{1/2} is shifted to ~ +20 ± 3 mV (mean ± SEM, n=10; Student's t-test, *, P=0.002). **b**, Wild type Prestin structure in a SO₄²⁻-based medium were obtained at three distinct configurations using single particle Cryo-EM technique. We considered two reaction coordinates (*z* and *d*) for determining the structural landscape of Prestin. *z* is the vertical distance between R399 backbone carbon and V499 backbone carbon (a residue on TM14 that resides on the protein-water interface; *d* is the distance between R399 backbone carbon and F137 backbone carbon which is equivalent to the binding pocket diameter, assuming the pocket as an sphere. All three residues are shown in Sticks. *z* and *d* has been measured across different states; Accordingly, from left to right, we have named the Prestin states: Down I, Down II, Intermediate (all SO₄²⁻) and Up (Cl⁻). The color code we have chosen for these states are red, yellow, blue and violet respectively. The right panel shows cross-sectional area expansion in Down (expanded) versus Up (compact) state. **c**, Movement of the anion binding site from Down to Up states. From Down to Up, first the electrical field becomes more focused due as the TM3 moves towards TM10 to form a helical dipole (i.e., the pocket becomes tighter, shown by the red arrow); this is followed by an elevator-like movement of the binding site from Down to Up, as shown by the cartoons and the yellow arrow. Down I, Down II and Intermediate are in the presence of SO₄²⁻, while in the Up state, Cl⁻ is the main anion. The structures were aligned based on residues 460 to 550 (TM13-TM14, the least mobile part of Prestin). For clarity, only TM14 has been illustrated. UCSF ChimeraX was used for illustration. **d**, The 1 μs all-atom molecular dynamics simulation of these states in POPC lipid bilayer reveals the dynamics of the anion-binding pocket as well as the water hydration (cyan) at different states. The number of waters in the intracellular cavity of Prestin reduces from Down to Up states (left to right). In addition, the pocket is the most apart in the Down I state (weak anion binding) and most confined in the Up (Cl⁻) state. For clarity, only the water molecules within 8 Å of the residues Q97, F101, F137, V397, S398, R399, E280 and E404 have been screened and illustrated using VMD.

Fig.3| Structural basis of Prestin inhibition by salicylate, and the evolutionary origins of electromotility. a, 10 mM salicylate inhibits Prestin function and flattens NLC and charge transfer when expressed in HEK293 cells (whole cell patch clamp electrophysiology, $n=7$; Student's t -test, $* P=0.005$).

b, Resolved structural densities indicates salicylate (shown in red) bound to anion pocket of Prestin, located at the core-gate interface. **c**, A zoomed inset of the anion-binding site formed by residues Q97, F101, F137, L397, S398 and R399 (shown in yellow), situated in the space confided by TM1 helix and the micro-dipole of TM3 and TM10. An overlay of the EM densities for sensor Up (Cl^-) versus Inhibited I (Cl^- +salicylate, left panel). For salicylate to be able bind to the pocket, TM10 helix should move downward to create enough space for accommodating the larger size salicylate compared to Cl^- . Salicylate molecule and density has been shown in salmon color. **d**, The Inhibited I (Cl^- +salicylate) and Inhibited II (Cl^- +salicylate) structures and the intermediate state are overlapping at the anion-binding site, (TM13-TM14 helices were aligned). The major difference between them is at the TM6-TM7 helical region of the core domain. The TM6 helix is kinked in the inhibited II state (yellow) causing this conformation to be further expanded. UCSF ChimeraX was used for the illustration. **e**, Evolutionary sequence alignment of TM6 in Prestin across different species. The focus is on the glycine residues that may underlie the flexibility of this helix during its motor function. **f**, Mutation of evolutionary conserved glycine residues such as G274 and G275 has major consequences on NLC and charge transfer. Double mutation of these two consecutive glycine residues diminishes electromotility. In addition, GG to GI mutation completely removed the NLC, while GG to IG mutation only left-shifted the NLC to -114 ± 3 (n=16; One way Anova, *, P=0.005). Mutation of other conserved Glycine residues along the TM6 helix shows their effects on the Prestin NLC and $V_{1/2}$ (n>12; Student's t-test, *, P=0.01). Mutation G270V the residue only conserved in mammals left shifts the NLC to -75 ± 0.5 (n=12), while other G263V and G265A almost completely abolishes the NLC.

a **b**

Fig.4| Structural basis of Prestin's voltage sensitivity and somatic electromotility. a. Complete set of Prestin structures stabilized by, either using anion binding as a proxy for shifts in the midpoint of the NLC ($V_{1/2}$), or stabilizing overall conformational dynamics by binding salicylate. Each experimental condition is represented by a cartoon, with its core domain (in blue) adjacent to the gate and intracellular domains. Transitions between states depend on the binding/displacement of anions or salicylate. The TM3-TM10 macrodipole gap is represented by two yellow cylinders encompassing the anion binding pocket. **b.** Structural transition of Prestin monomer from Down to Up conformation upon membrane depolarization (left to right, top panel). Anions are attracted to the focused positive potential field of the anion-binding pocket, which acts as “voltage sensor”. Upon depolarization, the voltage sensor moves from down to up, which is accompanied by an eccentric contraction of the Prestin's inter-membrane cross-sectional area as well as a major increase in the hydrophobic thickness of the protein (exaggerated cartoon). If the plasma membrane follows the footprint of Prestin during the process of Prestin charge

transfer, that means that the plasma membrane would thicken and the cell contracts upon membrane depolarization. This is consistent with the OHC movement (contraction) upon applying depolarizing potentials⁵⁴. Moreover, salicylate physically occludes the pocket and inhibits the voltage sensor movement; therefore, it inhibits Prestin's charge transfer and electromotility.

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