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Prestin at Year 14: Progress and Prospect

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Abstract

Prestin, the motor protein of cochlear outer hair cells, was identified 14 years ago. Prestin-based outer hair cell motility is responsible for the exquisite sensitivity and frequency selectivity seen in the mammalian cochlea. Prestin is the 5th member of an eleven-member membrane transporter superfamily of SLC26A proteins. Unlike its paralogs, which are capable of transporting anions across the cell membrane, prestin primarily functions as a motor protein with unique capability of performing direct and reciprocal electromechanical conversion on microsecond time scale. Significant progress in the understanding of its structure and the molecular mechanism has been made in recent years using electrophysiological, biochemical, comparative genomics, structural bioinformatics, molecular dynamics simulation, site-directed mutagenesis and domain-swapping techniques. This article reviews recent advances of the structural and functional properties of prestin with focus on the areas that are critical but still controversial in understanding the molecular mechanism of how prestin works: The structural domains for voltage sensing and interaction with anions and for conformational change. Future research directions and potential application of prestin are also discussed.

Keywords

outer hair cell; motility; prestin; SLC26a

1. Outer Hair Cell Motility

In 1985, Brownell and colleagues discovered that isolated outer hair cells (OHCs) from the guinea pig cochlea were able to change their length when the cells were electrically stimulated by passing extracellular current along the cell: Hyperpolarization of the

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membrane led to elongation of the cell, while depolarization resulted in cell contraction (Brownell et al., 1985; Kachar et al., 1986). Subsequently, either “motility” or “electromotility” is used to describe such change in the length of cells. Immediately after motility was discovered, understanding its mechanism and role in cochlear mechanics has quickly become one of the most exciting areas in auditory research for two obvious reasons: First, motility was rapidly recognized as a potential mechanism for cochlear amplification, the phrase that was originally coined by Davis (1983); Second, OHC motility appeared to be operated on a novel mechanism that was completely different from conventional energy-dependent, actin-myosin-based contraction in muscle cells (Brownell et al., 1985; Kachar et al., 1986; Ashmore, 1987; Holley and Ashmore, 1988). The magnitude of motility is close to 4% of the cell length and the motile response is contraction-asymmetric and nonlinear with saturation at the directions of contraction and elongation (Ashmore, 1987; Evans et al., 1989; Santos-Sacchi, 1989; Hallworth et al., 1993). The maximal motility sensitivity (slope) is approximately 20 nm/mV (Ashmore, 1987; Santos-Sacchi, 1989). Motility is driven by transmembrane voltage instead of transmembrane current (Santos-Sacchi and Dilger, 1988; Iwasa and Kachar, 1989) and can be blocked by gadolinium and salicylate ions (Santos-Sacchi, 1991; Shehata et al., 1991; Tunstall et al., 1995). Motile responses of OHCs are accompanied by charge movement, which is reflected in nonlinear capacitance (NLC) (Ashmore, 1989; Santos-Sacchi, 1991), akin to the translocation of gating charges of voltage-gated ion channels (Armstrong and Bezanilla, 1977). NLC of OHCs is characterized by a bell-shaped dependence on membrane potential, with a peak between −70 and −30 mV (Santos-Sacchi, 1991). Further experiments suggested that motility is associated with structures in the lateral wall of the OHCs (Ashmore, 1987; Holley and Ashmore, 1988; Huang and Santos-Sacchi, 1994). The strongest evidence for a plasma membrane-based mechanism came from experiments where motility was still detectable from cells after their cellular content was degradated by internal tryptic digest (Kalinec et al., 1992). Furthermore, patches of membrane withdrawn into the patch pipette responded to hyperpolarized and depolarized voltage change with increases and decreases in membrane area. These lines of evidence strongly suggest that the force generation mechanism is driven by voltage-dependent conformational changes of a molecular motor in the plasma membrane. In fact, Gulley and Reese (1977) already showed that the basolateral wall of OHCs contained a high density of intramembrane particles. These densely populated particles covered as much as 70% of the surface area on plasma membrane. The diameter of the particles was between 12 and 15 nm and the density was as high as 6,000 particles per square micrometer (Forge, 1991; He et al., 2010).

A distinct feature of OHC motility is its high speed. Measurements made from isolated OHCs or from membrane patches both show that the electromotile response occurs at microsecond time scale and works in a cycle-by-cycle mode up to a frequency of 20 kHz (Dallos and Evans, 1995; Gale and Ashmore, 1997; Frank et al., 1999). Although it is still controversial whether cycle-by-cycle OHC motility can occur at high frequencies *in vivo* due to the low-pass filter characteristic of the OHC basolateral membrane (Santos-Sacchi, 1992), it is quite certain that the motor itself has the ability to change conformation at high rate. It was also shown that voltage-driven motility is accompanied by a voltage-dependent change in axial stiffness (He and Dallos, 1999, 2000), although voltage-dependent stiffness

was not observed in another study (Hallworth, 2007). Voltage change can modulate cell stiffness over a range of about 10-fold and the overall stiffness of OHCs is reduced to ~1/3 of its normal value when motility is blocked (He et al., 2003). The force produced by a guinea pig OHC ranged from 20 to100 pN/mV (Hallworth, 1995; Iwasa and Adachi, 1997). Finally, OHCs demonstrate piezoelectric properties similar to a piezoelectric transducer (Iwasa, 1993; Gale and Ashmore, 1994; Ludwig et al., 2001; He et al., 2010). The efficiency of conversion from mechanical force to electrical charge is estimated to be ~20 fC nN^{-1}, four orders of magnitude greater than the efficiency of the best man-made piezoelectric material (Dong et al., 2002).

2. Discovery of Prestin

Two pieces of information before the year of 2000 played an important role in designing strategies to identify the elusive motor protein: First, electromotility is unique to OHCs, while inner hair cells (IHCs) are not electromotile; Second, the expression of electromotility is functionally detectable in gerbil OHCs starting from 6–7 days after birth (He et al., 1994; He, 1997). The onset of motility coincides with a significant increase in density of intramembrane particles in neonatal gerbil OHCs (Souter et al., 1995). These two lines of evidence indicate that the motor protein expression occurs after birth in altricial rodents. Two thousand IHCs and OHCs were isolated from gerbil cochleae and cDNA libraries were constructed for each cell type. An OHC subtracted cDNA library was subsequently produced to identify genes preferentially expressed in OHCs. Fifteen distinct genes were identified. Of these, one corresponded to an open reading frame of a protein containing 744 amino acids with a molecular mass of 81.4 kDa. The ontogenic expression of this cDNA was consistent with development of motility and intramembrane particles. When expressed in human embryonic kidney TSA201 cells, the resulting protein reproduced all hallmarks of the motor protein including voltage-dependent charge movement and cell motility (Zheng et al., 2000). The protein was named “prestin” to reflect the distinct feature of its ability to change conformation at high rate (prestin is from the presto musical notation). Antibodies generated against prestin detected prestin along the basolateral membrane of OHCs, showing a developmental expression pattern coinciding with the development of NLC and motility (Belyantseva et al., 2000). Subsequent experiments using prestin-null mice confirmed that targeted deletion of prestin resulted in loss of OHC electromotility and 40–60 dB loss of cochlear sensitivity (Liberman et al., 2002). Deletion of prestin also led to loss of voltage-dependent stiffness and piezoelectrical property of OHCs as well as significant reduction of the density of intramembrane particles in the plasma membrane (He et al., 2010). Taken together, all the evidence confirms that prestin indeed is the motor protein of cochlear OHCs.

3. Prestin Structure

Prestin shares the overall domain structure of the SLC26A protein family: a highly conserved central core of hydrophobic amino acids (~400 amino acid residues) with the N-terminal (~100 amino acid residues) and C-terminal (~240 amino acid residues) located in the cytoplasmic side of the plasma membrane (Fig. 1A). The sulfate transporter (SulTP) signature sequence is in the hydrophobic core, while a STAS domain (amino acid residues

510–710) with multiple clusters of charged residues is located in the C-terminal region (Oliver et al., 2001; Zheng et al., 2001). Analysis of prestin's structure with a number of topology prediction programs all indicates that the molecule has an even number of transmembrane (TM) domains (Fig. 1B). Based on immunocytochemical techniques using antibodies against different intracellular and extracellular domains of prestin and using hemagglutinin tag, a 12 TM domain topology model was proposed (Oliver et al., 2001; Zheng et al., 2001). However, Navaratnam and colleagues showed that prestin has only 10 TM domains based on their immunocytochemistry studies (Navaratnam et al., 2005). The main difference between the two topology models lies in the TM domains 5, 6, 7, and 8. Amino acids in the SulTP domain are almost completely identical among human, mouse, rat and gerbil prestins (only one amino acid is different). Sequence variations among mammalian species are primarily restricted to both the N- (residues 1–65) and C- (residues 516–744) termini with the C-end exhibiting the greatest variation. A relatively high amino acid sequence similarity is also shared by other mammalian species, including the prototherian platypus and the metatherian opossum (Okoruwa et al., 2008).

The STAS domain shares 10–15% sequence similarity with bacterial anti-sigma factor antagonists such as SpoIIAA of *Bacillus subtilis*. The STAS domain is conserved within related SLC26 gene family members including prestin. The STAS domain has a few structural features of interest that are based on the sequence alignment (Aravind and Koonin, 2000) and crystal/solution structures of SpoIIAA (Kovacs et al., 1998; Matsuda et al., 2004): it consists of four β-strands, five α-helices, a highly conserved loop that contains G/ATP binding sites and serine phospohorylation sites, and a variable loop. The G/ATP binding sites are consistent with the role of intracellular ATP in eliciting conformational changes (Aravind and Koonin, 2000). The serine residue in the conserved loop, a target for kinases and phosphotases for critical regulation of activity of SpoIIAA, is also present in the SLC26-associated STAS domain.

Experiments were performed to examine the importance of the N- and C-termini in prestin function. Deletion of either the N- or C- terminal region was shown to cause loss of membrane expression. There is conflicting evidence of whether the loss of function in truncated proteins is due to a lack of membrane insertion (Zheng et al., 2005; Navaratnam et al., 2005). Successive truncations of the amino acid residues in the C- or N-terminus resulted in a graded decrease and eventual elimination of NLC. In truncations that remained functional, voltage sensitivity (z) did not vary significantly from control values, indicating that the voltage sensor of truncated proteins remained relatively unaltered and that the voltage sensor was not in the regions that were truncated. Deletions of more than 21 amino acids from the N-terminus, or more than 32 amino acids from the C-terminus result in loss of prestin function (Navaratnam et al., 2005). Replacing the amino acids from either residue 515 or 644 at the C-terminal region of prestin with the corresponding C-terminal fragment of one of two closely related SLC26A proteins, Pendrin (SLC26A4) or PAT1 (SLC26A6), respectively, resulted in loss of membrane expression (Zheng et al., 2005). Mutating some of the non-charged amino acids in the C-terminal region led to either loss of membrane expression of the mutant proteins (such as Y52A or Y520A) or significant reduction of NLC without affecting membrane targeting (e.g., the double mutant V499G/Y501H prestin)

(Zheng et al., 2005). Taken together, these studies suggest that the C-terminal region plays an important role for both membrane targeting and overall function of prestin (Zheng et al., 2005), while N-terminal region is necessary for the formation of multimers as well as for membrane targeting (Navaratnam et al., 2005).

The question is then which domain mediates voltage sensing for prestin: the hydrophobic core region (SulTP domain) or the two terminal regions? Schaechinger and colleagues switched the SulTP domain between rat prestin and zebrafish prestin (Schaechinger et al., 2011). Zebrafish prestin is an electrogenic anion transporter that only exhibits minimal NLC (Albert et al., 2007; Tan et al., 2011). After swapping, the chimera containing the SulTP domain of rat prestin with N- and C-terminal regions from zebrafish prestin displayed robust NLC with voltage-dependent characteristics similar to that of rat prestin. Conversely, the chimeric zebrafish prestin with the N- and C-terminal regions swapped by those from rat prestin did not exhibit NLC. These studies indicate that NLC is mediated by the SulTP domain without the involvement of the cytoplasmic terminal regions.

4. Structure comparison among prestin's orthologs and paralogs

With major efforts in sequencing the entire genomes from a wide number of species, the deduced peptide sequence of a large number of mammalian SLC26A5 genes are now available with over 45 species currently being represented. In addition, prestin peptides were deduced from cDNAs that were obtained from over 25 species of bats, dolphins, porpoises and whales (Li et al., 2008; Liu et al., 2010; Davies et al., 2012; Shen et al., 2012). The number of corresponding sequence data for the SLC26A5 genes from avian and reptilian species has increased greatly in the last several years. Comparative amino acid sequence analyses have demonstrated that the SulTP and STAS domains are highly conserved with the greatest residue variations occurring in the N- and C-terminal regions.

In general, prestin has evolved from being quite diverse among extant species especially among the non-therian vertebrates (Franchini and Elgoyhen, 2006; Okoruwa et al., 2008). As depicted in Figure 2, amino acid sequences of prestin are quite variable among vertebrate species representing the bony fish, amphibians, reptiles and birds. Although frog prestin is the only amphibian representative, prestin peptide sequences are now available from a reasonable number of species in Sauropsida lineage (reptiles and birds). Prestin sequence diversity for the reptiles and birds is similar to that found among the bony fishes. A significant reduction in heterogeneity of the prestin sequence occurred in the divergence of the mammalian lineage. Comparative peptide sequence analyses demonstrate that mammalian prestins are much conserved with only minor changes occurring. Some sequence disparity is still present among the monotremes (prototherian species – platypus), marsupials (metatherian species – opossum, Tasmanian devil, and wallaby) and the eutherians. Among the clades of mammals, there is a relatively high amino acid sequence similarity where the eutherian SLC26A5 consensus sequence shares 79% and 83% residue identity to the platypus and opossum prestins, respectively (Okoruwa, *et al*., 2008). The platypus and opossum prestins exhibit an 81% sequence identity. However, the eutherian prestin is relatively invariant as demonstrated by having 98% sequence identity among four different mammalian species: human, mouse, rat and gerbil (He et al., 2006; Okoruwa et al.,

2008; Tan et al., 2012). This relative invariance is even more remarkable when compared to prestin's closest paralog, SLC26A4 (pendrin), which has 40% sequence identity with prestin (Dallos and Fakler, 2002; Mount and Romero, 2004; Tan et al., 2012). In comparison to prestin the other SLC26A paralogs do not exhibit such a high degree of conservation among eutherian species. The high conservation in eutherian prestin suggests that it has evolved in order to fit special mammalian needs (Franchini and Elgoyhen, 2006; Okoruwa et al., 2008).

Previous studies have demonstrated that insertions and deletions (indels) can contribute to the acquisition of motor-like properties in mammalian prestin (Okoruwa et al., 2008). There is a dichotomy between the therian/mammalian and non-mammal vertebrate sequences in four indel sites. There are four major segments containing indels in the amino acid sequences that are distinct to gerbil prestin from prestins of lizard, frog and bird. These indels are located with the following regions: one is associated with the mammalian motif (Okoruwa et al., 2008; Tan et al., 2012), two sites are within the charged cluster domain and the fourth is at the end of C-terminus. Even with the greatly expanded analyses of non-mammalian and mammalian species, these indel differences have remained as hallmarks of prestin diversity. It is unlikely that the indel site within the C-terminal region of the prestin has any effect on prestin function, since truncation of these residues does not alter NLC and plasma membrane targeting (Zheng et al., 2005). The two sites within the charged cluster domain may influence the folding of the positively and negatively charged clusters of amino acid resides as well as the spatial orientation of the STAS domain within the cytoplasm and the SulTP domain. In general, these differences are likely an indication of a significant transition of prestin from an electrogenic anion transporter to a unique molecular motor during evolution.

Based on differences in both amino acid sequences and the electrophysiological characteristics, a major episodic change(s) in prestin occurred with evolution of prestin in eutherian species, and also in the prototherians (platypus and echidna) and metatherians (e.g., opossum). The gain of motor capability is accompanied by diminishing transport function during evolution (Tan et al., 2011). The acquisition of motor properties in mammalian prestin also coincided with the cytoarchitectural changes in the inner ear with the evolution of an organ of Corti from the basilar papilla of birds and reptiles as well as in specialization of the lateral wall of OHCs in mammals (Manley, 2000; Manley and Fuchs, 2011). The phylogeny of the evolution of the organ of Corti and the specialization of inner and outer hair cells is shown in the insert of Figure 2. Subsequent adaptive changes also appear to have occurred in prestin with the evolution of echolocation in various bat and aquatic mammalian species (Li et al., 2008; Liu et al., 2010; Davies et al., 2012; Shen et al., 2012), but definitive evidence of prestin contributing to echolocation is still lacking.

5. Molecular mechanism of how prestin works

Although prestin was identified 14 years ago, still no experimental three-dimensional (3D) structure is currently available for it. This situation, in part, reflects the current lack of information about many integral membrane proteins, and so far, no member of the SLC26A family has been crystallized. Due to lack of knowledge of the 3D structure of prestin, most of our current knowledge about the mechanism of action of prestin is from experiments

using site-directed mutagenesis and/or domain-swapping techniques after the amino acid residues and/or domains were identified by comparing the sequence of prestin to those of its paralogs and orthologs.

5.1. Voltage Sensor

Prestin can sense external voltage and undergo conformational change, conceptually in a similar manner a voltage-gated ion channel can. Voltage-gated potassium channels, for example, are formed by four subunits. Each subunit comprises six transmembrane segments, S1–S6. The first four transmembrane segments, S1–S4, constitute the voltage sensor domain while the last two transmembrane segments, S5 and S6, form the pore for K^+ permeation (Bezanilla, 2000). S4, the voltage sensing helix, has many positively charged residues; therefore, a high positive charge outside the cell repels the helix, keeping the channel in its closed state. Depolarization of the cell interior causes the helix to move, inducing a conformational change that allows ions to flow through the channel (the open state). It is speculated that prestin might have similar structural domains that serve as a voltage sensor and an actuator. What is the voltage sensor and how does it work are two important questions that have instigated a lot of research since 2000. The first experiments that attempted to address these questions were performed by Oliver and colleagues (Oliver et al., 2001). Since pendrin, the closest paralog of prestin with 40% identity in amino acid sequence, exhibits no voltage-dependent NLC, it was reasonable to assume that the voltage sensor of prestin was made up of a charged residue present in the prestin sequence but absent in pendrin. A total of 21 non-conserved negatively or positively charged residues in the putative membrane domain of the prestin molecule were identified and mutated to neutral amino acids, glutamine or asparagine, either individually or in groups. In all cases the NLC, which reflects the voltage sensor, was still detected, although the peak of NLC was significantly shifted along the voltage axis in some mutants. The slope factor characterizing the voltage dependence was not significantly different between wild type and any of the mutants tested. The fact that the NLC could not be abolished by mutagenesis led to the idea that, instead of being an intrinsic property of the prestin molecule, the voltage sensor may be a charged particle extrinsic to the protein. Oliver and colleagues then replaced cations and anions on extra- and intracellular side of the membrane either by *N*-methyl-D-glucamine or tetra-ethyl-ammonium and pentane-sulfonate or sulfate, respectively. They showed that NLC and motility were abolished or significantly reduced only when intracellular anions were removed or replaced by pentane-sulfonate. Thus, it appears that intracellular anions, mainly Cl^- ions, play an important role in prestin function. These results led them to propose a model in which intracellular anions act as extrinsic voltage sensors, which bind to the prestin molecule and thus trigger the conformational changes required for motility of OHCs. According to this model, the anions binding to a site with high affinity are translocated across the membrane (manifested as nonlinear capacitance) by the transmembrane voltage: toward the extracellular surface upon hyperpolarization, and toward the cytoplasmic side in response to depolarization (Oliver et al., 2001; Dallos and Fakler, 2002). At present, it remains open whether, under physiological conditions, a single Cl^- ion is translocated per prestin molecule and per transport cycle, as indicated by the slope of the NLC ($z \approx 0.75$, which is equivalent to a single charge moving through about 75% of

the transmembrane electrical field). Alternatively, two (or more) Cl^- ions might be translocated halfway (or less) through the membrane.

So far almost 200 amino acid residues have been mutated in order to search for the voltage sensing mechanism and for the amino acid residues that are critical for prestin function (He et al., 2006). Although a majority of the mutants continued to show NLC after the charged amino acids in the SulTP region were replaced with neutral amino acids, other mutations (A100L or A100V; A102L or A102V; G324W) were able to abolish NLC (Rajagopalan et al., 2006; Bai et al., 2009). These studies raise the question of whether the voltage sensor of prestin is indeed extrinsic, although the possibility of whether those mutations can alter the structure of transmembrane helices without changing membrane expression cannot be ruled out. Other studies also showed that some cysteine residues are associated with charge transfer, however, the mechanism is not clear (Bai et al., 2010a,b; McGuire et al., 2010).

Rybalchenko and Santos-Sacchi (2003a,b) examined the relationship between the conformational state of prestin and the effectiveness of anions on the electrophysiological activity of prestin and provided some new data that were not consistent with the extrinsic voltage sensor model proposed by Oliver and colleagues. Their results suggested that anions work as an allosteric modulator of the voltage sensor of prestin by the following reasons (Song and Santos-Sacchi, 2010): 1) changes in the intracellular anion concentration and species shift the voltage dependence of NLC; 2) prestin's susceptibility to many biophysical forces, and particularly its piezoelectric nature, may reflect anion interactions with the motor; 3) the anion valence does not correlate with the voltage sensor valence; and 4) intrinsic charges may contribute to the voltage sensing of prestin, because mutations of four negatively charged residues (D83, E293, E374, and E404) diminished NLC (Bai et al., 2009). On the bases of these evidences, a mechanism that is based on movement of charged or polar residues, modulated by Cl^- ions in an allosteric manner was proposed (Song and Santos-Sacchi, 2010). It should be pointed out that the model proposed by Oliver and colleagues also involves binding of anions to the prestin molecule (Oliver et al., 2006). Therefore, some of the evidence presented by Song and Santos-Sacchi (2010) can be explained by the extrinsic voltage sensor model.

No matter whether the intracellular anions are the extrinsic voltage sensor as proposed by Oliver and colleagues (Oliver et al., 2006) or function as allosteric modulator of the voltage sensor as first proposed by Rybalchenko and Santos-Sacchi (2003b), it is well accepted that intracellular Cl^- ions are critical for prestin function. Therefore, the knowledge of the identity of the amino-acid residues that are either structurally or functionally part of the voltage sensing is crucially important. Experiments from three different laboratories appear to have identified completely different residues and regions for voltage sensing. Zheng and colleagues identified amino acid residues 499 to 501, near the presumed junction between the last transmembrane domain and the intracellular C-terminal region, to be critical for the expression of NLC (Zheng et al., 2001). They showed that double mutations of V499G and Y501H resulted in significantly diminished NLC and motility (Dallos et al., 2008). These mutations appear to significantly impair the fast motor kinetics and voltage operating range (Homma et al., 2013). How these non-charged amino acid residues affect the function of prestin is unknown. They may interfere with voltage-regulated anion binding, or the

consequent conformational change of the protein. A new homology model of the transmembrane domain of prestin predicts that these two amino acid residues are at the intracellular entrance of the hypothetical channel that controls the entrance of anions to interact with other amino acids (Lovas et al., 2011).

Oliver and colleagues probed different regions within the 12 TM helices model of prestin for their functional significance (Schaechinger et al., 2011). They identified a region comprising the first two transmembrane helices (residues 86 to 140) of the rat prestin. This region exhibits the highest degree of sequence identity across the SLC26A transporter family and includes the highly conserved SulTP domain. When this region of rat prestin was replaced with the respective motif from zebrafish prestin, NLC was completely abolished, suggesting that this domain may contain structural domain essential for prestin function. Sequence comparison showed that only seven amino acids are different within this region between the rat and zebrafish prestins. After they replaced each of these seven residues in rat prestin individually by the respective amino acid of zebrafish, three residues (L93M, F101Y, P136T) appeared to be critical. Mutations of these three residues completely abolished NLC. They identified another segment comprising the putative TM helices 9 and 10 (residues 381–438 in the rat prestin sequence). On its own, this region was also not sufficient to confer zebrafish prestin with NLC. However, in combination with the N-terminal segment around TM domains 1 and 2, the chimeric zebrafish prestin generated robust NLC.

In contrast, another approach to predict the structure(s) responsible for the evolution of motor capability involves identifying region(s) that are new and conserved in mammalian prestin, but are highly variable in the non-mammalian vertebrate prestin orthologs. Using a comparative and evolutionary approach and consensus amino-acid sequence analyses, an amino-acid motif within the SulTP domain of prestin was identified (Okoruwa et al., 2008). This motif, comprising of 11 amino acids (residues 158 to 168), is extremely conserved among eutherian species but highly variable among non-mammalian orthologs and SLC26A paralogs (Tan et al., 2011). Corresponding residues from zebrafish and chicken prestins were swapped with the motif of gerbil prestin. Motility and NLC were measured from chimeric prestin-transfected HEK-293 cells using a voltage-clamp technique and photodiode-based displacement measurement system. A gain of motor function with both hallmarks representative of mammalian prestin function was observed in the chimeric prestin without loss of transport function. Hence, substitution of the identified motif for the corresponding residues confers the electrogenic anion transporters of zebrafish and chicken prestins with motor-like function. Thus, this motif represents the structural adaptation that assists gain of motor function in eutherian prestin (Tan et al., 2012). Pendrin receiving substitution of this motif in the respective region also gains motor function (Tang et al., 2011). Contrastingly, these two studies by Schaechinger and colleagues (Schaechinger et al., 2011) and Tan and associates (Tan et al., 2011) are fundamentally different in the way of how the motifs were identified. At present, it is difficult to reconcile these different conclusions, although, it is possible that there may be several areas in the prestin molecule that facilitate, *in toto*, the maximum gain of motor function.

5.2. Actuator and voltage sensor are coupled

Conceptually prestin should have at least two essential functional domains: the voltage sensor that detects changes in the membrane potential, and the actuator that undergoes a conformational change. Regardless whether the voltage sensor is intrinsic or extrinsic, anions need to be translocated across the membrane in response to changes in the transmembrane voltage: moving towards the extracellular surface following hyperpolarization, and towards the cytoplasmic side in response to depolarization. As a consequence, this translocation triggers conformational changes in the protein that ultimately alter its surface area in the plane of the plasma membrane. The area decreases when the anion is near the cytoplasmic face of the membrane ('short state'), and increases when the ion has crossed the membrane to the outer surface ('long state'). At the normal resting potential (-70 mV), the molecule is more likely to be in its long state. Consequently, the cell contraction caused by depolarization is greater than the cell elongation caused by hyperpolarization (Dallos and Fakler, 2002). The first attempt to detect the conformational changes of prestin was carried out using the fluorescence resonance energy transfer (FRET)-based technique (Gleitsman et al., 2009). Prestin was labeled with fluorophores at either the N- or C-terminal end in transfected HEK-293T cells, and FRET changes during membrane potential change was monitored. They detected a significant decrease in inter-subunit FRET between the C-terminal and N-terminal regions. Changes in FRET were suppressed by either salicylate treatment or by mutations at V499G/Y501H. These results are consistent with significant movements in the C-terminal domain of prestin upon change in membrane potential. Their studies have provided the first demonstration of the internal dynamic motion of the molecule. However, it is still unresolved as to where exactly the actuator is and how it works.

Experiments were also carried out to address the question of whether the voltage sensor and actuator are fully coupled. Wang and colleagues compared the voltage dependency and the values of slope factor of NLC and somatic motility simultaneously measured from the same OHCs (Wang et al., 2009). They showed that the voltage dependency and slope factors of NLC and motility overlap with identical values, suggesting that NLC and motility are fully coupled and that the charge movement is directly converted into conformational change of the prestin molecule. Using the same technique, Homma and Dallos examined motility and NLC under conditions in which the magnitudes of both charge movement and electromotility were gradually manipulated by the prestin inhibitor, salicylate (Homma and Dallos, 2011a,b). They showed that prestin-associated charge movement and the resulting electromotility are fully coupled and that prestin has at least two voltage-dependent conformational transition steps. While tight coupling of motility and NLC was also demonstrated in a recent study (Song and Santos-Sacchi, 2013), intracellular Cl^- concentration can significantly modulate the coupling.

5.3. Oligomerization

Protein oligomerization is thought to be an advantageous feature of evolutionary adaptation to further facilitate protein function (Ali and Imperiali, 2005). An example is the voltage-dependent channels that are formed by four subunits (potassium channels) to form a central aqueous pore for ion permeation. In other words, the 4 or 6 individual subunits are required

to work cooperatively in order to allow ions to permeate through the central aqueous pore formed by the subunits. Since oligomerization and cooperativity are important for ion channel function, the questions then are: Does prestin forms oligomers? If it does, is it a dimer, trimer, tetramer or higher-order oligomers? Is cooperativity among monomers necessary for prestin function?

Freeze fracture images of the OHC lateral wall plasma membrane have revealed a dense, regularly spaced array of 12-nm round particles (Forge, 1991; Kalinec et al., 1992; Souter et al., 1995; Koppl et al., 2004). However, the particles were 4–5 times the expected size of the 744-amino acid prestin. In order to reconcile the predicated size of the prestin peptide and the intramembrane particles in the OHC plasma membrane, prestin peptide was predicated to form oligomers. Evidence for the oligomerization of prestin comes from a number of studies using different approaches. Western blot analysis of prestin purified from in vitro expression methods suggested dimer and tetramer states, with the tetramer state being more easily dissociated (Zheng et al., 2006). However, another study found that dimers were the normal configuration of prestin as well as with other SLC26A family members (Detro-Dassen et al., 2008). Recent single particle structure analysis based on an electron microscopic image of purified prestin protein has shown that prestin is a bullet-shaped fourfold symmetric molecule with an inner cavity, suggesting a tetrameric arrangement of subunits (Mio et al., 2008; Murakoshi et al., 2009; Kumano et al., 2010). On the bases of charge density measurements of prestin-mutant OHCs and freeze fracture microscopy, calculations support the notion that prestin exists as tetramer in the membrane of OHCs (He et al., 2010). Prestin existence in tetrameric form is now also supported by evidence from experiments using FRET analyses of prestin (Hallworth and Nichols, 2012; Bian et al., 2013). Biochemical and imaging techniques further show that prestin forms homo-oligomers and that N-terminal residues mediate homomultimerization of prestin (Navaratnam et al., 2005; Bai, et al., 2010a; Currall et al., 2011). Disulfide bonds embedded through cysteine residue in the SulTP domain (Zheng et al., 2006; Bai et al., 2010a) or hydrogen bonding through residue C415 (Currall et al., 2011) have been proposed to be important for the oligomerization of prestin.

Is there cooperativity among prestin subunits? Perhaps the inheritance pattern of SLC26A-associated diseases in humans with Pendred Syndrome (Dossena et al., 2009) offers the best initial clue. Mutations in SLC26A4 were recessive and did not exhibit the dominant negative mutational effects as observed in the cases of mutations of voltage-gated potassium channels (Dror et al., 2011), indicating that each monomer is functionally independent. In general, transporters in the SLC26A family form multimeric proteins, but have functionally distinct subunits (Gouaux and Mackinnon, 2005). FRET analyses suggest the existence of homo-oligomeric interaction of prestin molecules (Navaratnam et al., 2005; Greeson et al., 2006; Gleitsman et al., 2009; Bai et al., 2010b). Detro-Dassen and associates found that prestin molecules formed in cells co-expressing two adjoining prestin mutations with different electrical properties had intermediate electrical properties in the aggregate (Detro-Dassen et al., 2008). This, they suggested, indicates that one subunit can influence another, which implies cooperativity. However, Wang and colleagues argued, based on simultaneous measurements of motility and NCL from isolated OHCs, that each subunit operates independently (Wang et al., 2009). They showed that the slope factor is the same between

NLC and motility, indicating that each prestin monomer in the tetramer is in parallel, each interacting independently. More evidence now supports the notion that each prestin subunit acts independently in the oligomer (Hallworth and Nichols, 2012; Bian et al., 2013; Homma et al., 2013).

5.4. Transport function

Paralogs of prestin and non-mammalian orthologs are anion exchangers/transporters or anion channels (Albert et al., 2007; Schaechinger and Oliver, 2007; Bai et al., 2009; Tan et al., 2011). It is important to determine whether prestin still retains transport function after gaining motor function. Oliver and colleagues showed that prestin is an incomplete transporter and unable to transport anions across the membrane (Oliver et al., 2001). Muallen and Ashmore (2006) used kinetic models to understand the mechanism. They showed that the model proposed by Oliver and colleagues, which assume prestin cannot operate as a transporter, was insufficient to explain previously published data. They proposed an alternative model of prestin being as an electrogenic anion exchanger, exchanging one Cl^- ion for one divalent or two monovalent anions. Bai and colleagues (2009) showed first that prestin can transport anions cross the membrane using classical radioactive anion uptake techniques. A potential Cl^--binding site was identified and mutations of two residues (P328A and L326A) associated with this site preserved NLC, yet negated anion transport capability. Their study suggests that prestin is still able to transport anion and that transport and voltage sensing are independent. Two other studied found that prestin can act as a weak $Cl^-/HCO3^-$ antiporter (Mistrik et al., 2012) or as a weak electrogenic anion transport (Schanzler and Fahlke, 2012). It is worth noting that recent studies using radioactive anion uptake techniques failed to detect anion uptakes in mammalian prestin (Tan et al., 2011; Tang et al., 2013). In spite of the fact that it is still controversial whether prestin retains transport function, it is quite certain that its transport capability is significantly diminished as prestin gains motor function in mammals during evolution (Tan et al., 2011; Schanzler and Fahlke, 2012). It is also generally accepted that transport function, if any, and motor function are independent with separate structural basis (Bai et al., 2009; Tang et al., 2011; Tan et al., 2012).

5.5. Phosphorylation of prestin

Several phosphatases or kinases, including cGMP-dependent protein phosphorylase, have been shown to influence OHC electromotility (Szönyi et al., 1999; Frolenkov et al., 2000, 2001; Deak et al., 2005). It was also speculated that the effects of ACh on motility were mediated by phosphorylation pathways (Kalinec et al., 2000; Sziklai et al., 2001; Zhang et al., 2003). Prestin has several potential phosphorylation sites including two locations for phosphorylation by cAMP/cGMP-dependent protein kinase as predicted by PROSITE search (Bairoch and Apweiler, 1997). Deak and colleagues examined effect of cGMP on the function of prestin by generating nine mutations at putative phosphorylation sites of prestin, represented by the motifs: KRYS and KRKT, at the S238 and T560 positions, respectively (Deak et al., 2005). The neutral amino acid alanine was used to replace serine/threonine at phosphorylation sites to change the conserved phosphorylation motif. These alterations mimicked the dephosphorylated state of prestin. Replacement with the negatively charged aspartic acid was used to mimic the phosphorylated state. cGMP was demonstrated to be

significantly more influential than cAMP in modifying NLC. The electrical properties of the single and double mutations further indicate a possible interaction between the two PKG target sites. One of these sites may influence the expression of prestin on the membrane. In addition, these studies demonstrated that phosphorylation can influence motility.

6. Prospect

Prestin is a novel type of biological motor protein. Its mechanism is entirely different from the well-known and much studied classical cellular motor proteins such as myosin. The action of prestin is also orders of magnitude faster than that of any other cellular motor proteins. To fully comprehend the structure-function relationships of prestin, it is necessary to describe in atomistic details its conformational change as a function of time in response to the change in membrane potential. Based on the deletion and site-mutagenesis experiments it has become more apparent that appropriate folding of prestin is required for cell surface expression and the functional constraints for transporter and motor functions requires prestin *in toto*. Certainly some variation is tolerated as observed in evolution of prestin. However, the minimal diversity exhibited in eutherian prestin further supports the contention of the entire 3D structure of prestin as a critical requirement for motor function. Lack of structural information leaves it unclear what domains, motifs or residues of prestin are critical for the charge movement and for any structural changes it undergoes when either intrinsic or extrinsic charge movement is taking place. Among the numbers of recent attempts to identify critical regions of the prestin, a combination of comparative genomics, structural bioinformatics, molecular dynamics (MD) simulation methods, and mutagenesis studies seem to be promising. The method of MD simulations is one of the principal tools in the theoretical study of biopolymers. MD simulations can provide detailed information on domain movements, conformational changes and residue fluctuations of proteins and is widely used to investigate protein-protein and protein-ligand interactions. It is also used to fine tune structures from both X-ray crystallography and from nuclear magnetic resonance spectroscopy. Lovas and colleagues have begun to use MD simulations to study the structure- function relationships of prestin (Lovas et al., 2011). They determined the 3D model of the SulTP domain of rat prestin (Fig. 3) by combining *ab initio* structure prediction, 3D-folding recognition by threading (Jones, 1999), homology modeling and MD simulations. They showed that the structure of prestin is similar to that of the bacterial glutamate/aspartate transporter Glt_{Ph} (Yernool et al., 2004) with the major structural features of 8 TM spanning domains, TM3-TM4 extracellular loop, two helical pin re-entry loops and intracellular finger (ICF). Unique feature of the proposed structure is the ICF (residues 133–156) which extends into the intracellular regions and during MD simulations it formed a stabile antiparallel β-sheet structure. HEK394 cells expressing the variant of the rat prestin from which the intracellular finger region was deleted had substantially decreased NLC, indicating that intracellular finger is still needed for the full biological activity of prestin. This supports the proposed structure by Lovas and colleagues that ICF is not part of the TM region helices of prestin and the protein still can fold into a cell membrane-expression structure.

X-ray crystallography is one of the most important techniques in determining the 3D structure of a molecule. Complete understanding of the molecular mechanism of how prestin

works requires a 3D structure from X-ray crystallography. Although crystallizing the entire prestin or the SulTP domain is still a significant scientific/technical challenge, an attempt was made to examine the 3D crystal structure of the C-terminal STAS domain (residues 505–718) of prestin (Pasqualetto et al., 2010). Since the domain contains a variable and structurally flexible loop region (residues 564–636), only a shorter variant ([505–563]GS[637–718]) was crystallized. The structure composed of six β-sheets and five α-helices with an overall ovoid topology. STAS domain of prestin shares low sequence similarity to bacterial anti-sigma factor antagonists such as SpoIIAA of Bacillus subtilis, however, the STAS domain is conserved within related SLC26A family members and among prestin sequences in many different species. The low sequence similarity between bacterial and prestin STAS domains is expressed in their 3D structural differences, especially at the N-terminal region (Pasqualetto et al., 2010). Furthermore, these authors proposed that a non-polar surface region of the STAS domain most likely interacts with the cell membrane and that the N-terminal region is in close proximity of the membrane, whereas the C-terminal end is immersed in the cytosol. Although Pasqualetto and colleagues were only able to examine the 3D structure of a partial region of the C-terminal STAS domain, their study represents the first attempt to examine the 3D structure of prestin using X-ray crystallography.

The lack of crystallization of the STAS domain in the presence of the variable and structurally flexible loop region illustrates very well that beyond experimental techniques theoretical studies that provide 3D structure of proteins at atomistic details are necessary. Homology modeling combined with subsequent MD simulations for structure refinement offers such opportunity. Furthermore, Bai and colleagues identified three charge clusters in the C-terminal region of prestin (Bai et al., 2009). Two of these charge clusters are in the flexible loop region. Although neither charge cluster mutants showed diminished NLC, the first and the second cluster (residues 608–618) shifted voltage-dependence, clearly indicating that the 3D structure of this region should be examined in order to explain their role in the functional properties of prestin.

Prestin shows a real promising potential for future nanotechnology applications. Because of its speed and piezoelectric properties, prestin ensembles could function as mechanical, voltage-controlled actuators with exceptional speeds. Prestin could also serve as a sensor of mechanical stress, or operate as a voltage-controlled capacitor in electrical nanocircuits. Understanding how prestin works and identifying the amino acid residues and either motifs or larger domains that constitute the structural basis for motor function can help to design and create nanomotors for a variety of applications in medicine, protein and mechanical engineering, and even in the defense industry. Identifying the essential motifs and domains for voltage sensing and motor capability is the first step for synthesis and modification of the molecule (Schaechinger et al., 2011; Tang et al., 2011).

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Abbreviations

3D	three-dimensional
FRET	fluorescence resonance energy transfer
IHC	inner hair cell
MD	molecular dynamics
NLC	nonlinear capacitance
OHC	outer hair cell
SulTP	sulfate transporter
STAS	sulfate transporter and antisigma-factor antagonist
TM	transmembrane

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Highlights

- We review basic properties of OHC electromotility
- We describe how prestin was discovered
- We review current view of the structure of prestin
- We review recent advances of the molecular mechanism of how prestin works
- We discuss the prospect of future research of prestin

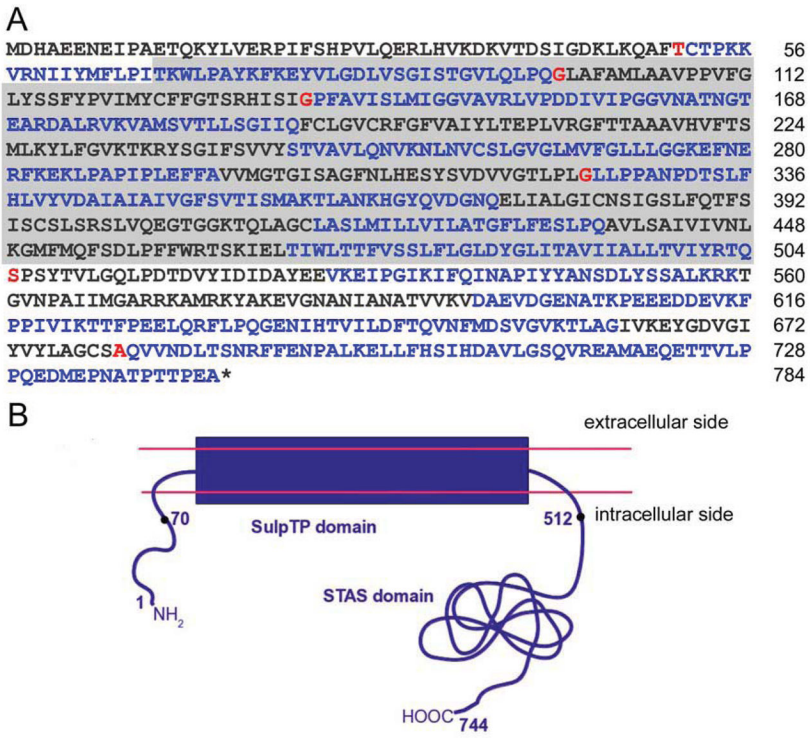

Figure 1.

Amino acid sequence and topology model of prestin (rat). **A**: Amino acid sequence is provided with the exon organization indicated by alternating black and blue text colors. Codons that are shared between exons are demarked in red. The gray shaded amino acids represent the SulTP domain. **B**: A generic topology model with both N- and C-termini intracellularly. The two amino acids (70 and 512) define SulTP domain from the two termini.

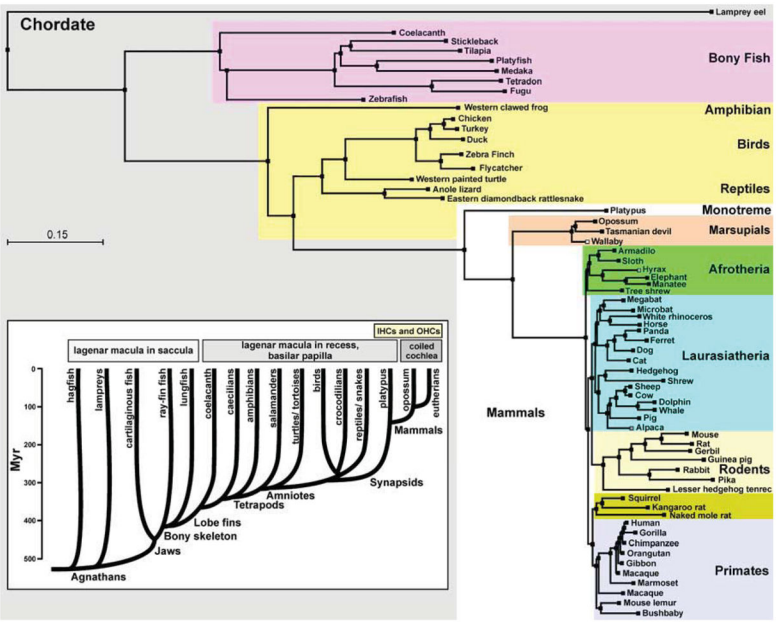

Figure 2. Cladogram of the evolution of prestin

The tree of evolutionary relationship of vertebrate species was used to determine the evolution of prestin in vertebrates. The SLC26A5 gene of Lamprey eel was used the outgroup for the vertebrate orthologs of prestin and was used as the representative species of chordates. Peptide homology was determined using Muscle alignment analyses and the phylogenetic tree was determined using 500 and 1000 iterations (Okoruwa et al., 2008). Trees were established using MEGA5 program (Tamura et al. 2007) and CLC Main Workbench (http://www.clcbio.com/products/clc-main-workbench/) with the following parameters: neighbor-joining, pairwise deletion, constant substitution rate, and the amino acid JTT matrix. Reliable bootstrap values were obtained for all nodes of the tree except for the terminal nodes linking the lamprey eel prestin peptide. Stability of the clades was evaluated by 500–1000 bootstrap rearrangements. The *bar* on the tree represents the branch length equivalent to 0.15 amino acid changes per residue. The common names for species used in these analyses are provided and grouped into the non-mammalian vertebrates that include the bony fish, amphibians, birds and reptiles. These clades of mammals are the monotremes, marsupials, afrotheria, laurasiatheria, rodents and primates. These groups are colored coded. Peptides with partial sequence data (75–90% complete) are represented by the gray filled boxes. An insert is provided that depicts the phylogeny of the major groups of extant vertebrate through geologic time based on acquisition of distinctive morphological features. The relationship of phylogeny of inner ear morphology and the evolution of inner and outer hair cells is also provided (Manley, 2011; Fritzsch et al., 2013). The branch lengths are proportional to the length in millions of years (Myr) at the time of divergence of the taxa.

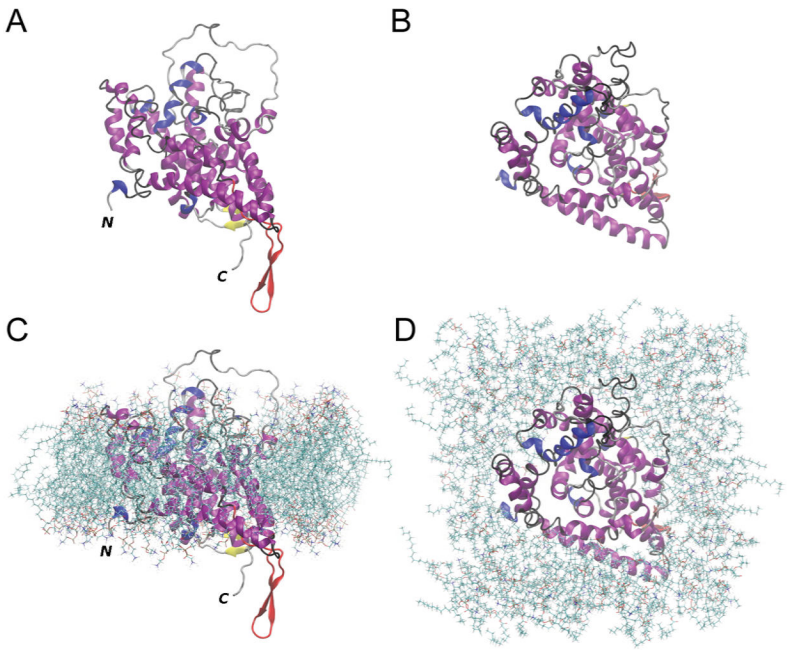

Figure 3.

3D structure of the SulTP domain of prestin. **A**: Side view of prestin in ribbon representation (top is the extra cellular region); purple, α-helix; blue, 3_{10}-helix; yellow, β-sheet; black, β-turn; grey, random meander. The intracellular finger is in red. ***N*** and ***C*** indicate the amino- and carboxy-terminal ends, respectively. **B**: Top view of prestin. **C**: Side view of prestin as embedded in a lipid bilayer. Top of the figure is the extra cellular region. **D**: Top view of prestin as embedded in a lipid bilayer.