Tension Sensitivity of Prestin: Comparison with the Membrane Motor in Outer Hair Cells

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ABSTRACT The membrane motor in outer hair cells undergoes conformational transitions involving charge displacement of ~0.8 *e* across the membrane and changes of ~4 nm^2 in its membrane area. Previous reports have established that the charge transfer in the membrane motor and that in prestin, a membrane protein in the plasma membrane of outer hair cells, are approximately equal. Here, we determine the membrane area changes based on its sensitivity to membrane tension. We found that prestin does undergo area changes and that the magnitude is ~1 nm^2, smaller than the value 4 nm^2 for outer hair cell motor. This result confirms that prestin is a protein that functions as a membrane motor based on piezoelectricity. The discrepancy in the magnitude could suggest a prestin-containing complex in outer hair cells.

INTRODUCTION

Prestin (Zheng et al., 2000) is a membrane protein unique to the lateral wall of outer hair cells. This protein is essential for the cells' voltage-dependent motility (electromotility) at their cylindrical cell bodies. Because outer hair cells have mechanotransducers at their hair bundles, the voltage-driven motor can provide positive feedback, leading to increased sensitivity of the ear (see Patuzzi, 1996, for recent review). The importance of prestin was demonstrated by a recent experiment that prestin knock-out animals indeed showed a significantly elevated hearing threshold (Liberman et al., 2002). These experiments do demonstrate that prestin is an essential part of the membrane motor in outer hair cells. A question that is still not fully clarified is whether prestin acts alone as a motor in outer hair cells or it is simply an important constituent of a protein complex that acts as the motor.

The membrane motor in outer hair cells has been characterized based on a two-state model with two parameters: charge that gains energy by moving across the membrane and membrane area changes that are associated with it. The mobile charge is between 0.7 and 1 *e* (Ashmore, 1990; Santos-Sacchi, 1991; Iwasa, 1993), where *e* is the electronic charge. The membrane area changes that are coupled with charge transfer are ~4 nm^2 (Iwasa, 1993; Adachi and Iwasa, 1999). This electromechanical coupling is consistent with piezoelectricity (Dong et al., 2002).

The voltage dependence of the membrane capacitance shows that prestin's mobile charge is ~0.9 *e* (Zheng et al., 2000; Oliver et al., 2001), similar to that of the membrane motor in outer hair cells. However, the mechanical changes that accompany charge movement has not been evaluated. Although the existence of prestin's tension sensitivity was reported by Santos-Sacchi et al. (2001) and by Ludwig et al. (2001), the sensitivity has not been determined. If prestin operates based on piezoelectricity, we should be able to determine the area change coupled to charge transfer that characterizes the molecule as a motor (Iwasa, 2001).

To examine mechanical changes in prestin that accompany charge transfer, we apply known membrane tension to prestin-transfected cells by delivering pressure through the patch pipette. If the cell is fully inflated and the shape of the cell is supported by the plasma membrane, membrane tension can be estimated by the cell radius and applied pressure, assuming Laplace's law. The tension sensitivity of the voltage-dependent membrane capacitance then leads to area changes of the motor (Iwasa, 1993; Adachi and Iwasa, 1999). If the area change of prestin thus determined is similar to the area change of the motor in outer hair cells, prestin is the membrane motor in outer hair cells. If the difference is significant, the membrane motor in outer hair cells is likely a complex of membrane proteins involving prestin.

MATERIALS AND METHODS

Prestin-transfected cells

A human embryonic kidney cell line, 293T was used for transiently expressing prestin. We followed the method that was described by Zheng et al. (2000). Briefly, the cells were cultured in a 35-mm culture dish filled with Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were cotransfected with prestin-encoding plasmid pcDNA3.1, which is provided by Dr. L. Madison of Northwestern University, and green fluorescent protein (GFP)-encoding plasmid. We used Ca phosphate as the transfection reagent (CalPhos Mammalian transfection kit, BD Biosciences, San Jose, CA). Approximately 80% of the cells that expressed GFP had voltage-dependent capacitance, an indicator of prestin in the plasma membrane. Experiments are performed on those cells between 24 and 72 h after transfection.

Membrane capacitance measurement

Patch pipettes were manufactured with a pipette puller (Model 81, Sutter Instruments, Novato, CA). The resistance of the pipettes used was between 2 and 3 MΩ when filled with an intracellular medium (see below). Experiments were performed at 22°C.

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The internal solution consisted of 140 mM CsCl, 2 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, and 0.11 mg/ml trypsin (type I, Sigma, St. Louis, MS). The external solution contained 140 mM NaCl, 5 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $CoCl_2$, 10 mM HEPES, and 10 mM glucose. These channel-blocking media facilitated capacitance measurement. The osmolarity of each medium was adjusted to 300 mOsm/kg with glucose. In all media the pH was adjusted to 7.4. A patch amplifier (Axopatch 200B, Axon Instruments, Union City, CA) was used for whole-cell voltage clamp experiments. A train of voltage pulses was generated with an ITC-16 interface (Instrutech, Great Neck, NY) using the Igor program (Wave Metrics, Lake Oswego, OR) with a software module created by R. J. Bookman's laboratory at the University of Miami (http://chroma.med.miami.edu/cap/).

The membrane capacitance was determined by capacitive currents elicited by voltage jumps, using the method described in a previous report (Adachi and Iwasa, 1999). To scan the voltage range from −120 mV to 50 mV, we used a staircase waveform that had 10-mV jumps at 3-ms intervals. The first part of the waveform consisted of upward jumps and the second part was downward jumps. The sampling interval of the data acquisition was 10 μs. The relaxation time τ of the capacitive currents was somewhat >200 μs. The access resistance R_a was (8.6 ± 2.7) MΩ. The membrane resistance R_m was somewhat dependent on the membrane potential and was between 0.2 GΩ and 1.5 GΩ. At the extremes of the membrane potential, R_m was (0.4 ± 0.1) GΩ and was (0.9 ± 0.5) GΩ in the middle range. The membrane potential was corrected for the access resistance, although the corrections were insignificant in most cases.

The membrane capacitance $C_m(V)$ was described by a derivative of the two-state Boltzmann function that involves phenomenological mobile charge $\hat{q}$, the phenomenological number of charges $\hat{N}$ in the cell, and the peak potential V_{pk},

$$C_m(V) = C_{lin} + \frac{\hat{N}\hat{q}^2}{k_B T} \cdot \frac{B(V)}{(1 + B(V))^2}, \quad (1)$$

with

$$B(V) = \exp[\hat{q}(V - V_{pk})/k_B T]. \quad (2)$$

Here, C_{lin} is the linear capacitance, k_B the Boltzmann constant, and T the temperature.

Pressure application

Positive pressure was applied to the pipette through a solenoid valve (General Valve, Fairfield, NJ), which was connected to a cylindrical reservoir with a piston. Pressure was adjusted by changing the weight placed on the piston. The pressure at the reservoir was monitored with a pressure gauge (model 68370, Cole-Parmer, Vernon Hills, IL).

Images of cells under the experiment were saved in a video recorder and digitized off-line. Video images of the cell obtained with Hoffman differential contrast optics (Modulation Optics, Greenvale, NY) on a Nikon microscope were recorded during the experiment and digitized off-line with the National Institutes of Health Image program (W. Rasband, http://rsb.info.nih.gov/). The resolution of the digitized image was 4.04 pixels per μm.

RESULTS

To be able to use Laplace's law for estimating membrane tension, two conditions need to be met. First, the plasma membrane is not supported by the cytoskeleton. Second, the cell is fully inflated. To achieve the first condition, we digest prestin transfected cells from inside. To achieve the second condition on trypsin-treated cells, we found that we needed to adjust the holding potential.

Internal digestion by trypsin

To remove possible effects of the submembranous cytoskeleton, we internally treated transfected cells with trypsin. After the establishment of whole-cell recording configuration, we injected trypsin-containing (0.1 mg/ml) pipette medium into the cell by gentle pressure applied with a hypodermic syringe.

On injection of trypsin-containing medium, the plasma membrane detached from the cytoskeleton and radius of the spherical cell increased by ~30% (Fig. 1). The contrast at the plasma membrane decreased with expansion. This process was irreversible. The removal of applied pressure does not lead to recovery in the cell radius. Such an effect of trypsin injection is similar to that in outer hair cells, in which dissolution of cytoskeletal elements is confirmed with electron microscopy (Huang and Santos-Sacchi, 1994).

The radius of internally digested cells underwent gradual changes depending on the holding potential. When the holding potential was more negative than ~−77 mV, the radius of the cell remained approximately constant. However, the radius gradually decreased when the holding potential was more positive, accompanied by an increase in the contrast at the fringe of the cells. Such a change was not evident when the cytoskeleton was not treated with a protease.

Peak shift of fully inflated cells

The membrane capacitance was determined by recording currents elicited by a series of a staircase voltage waveform. Because the voltage-dependent component of the membrane capacitance was rather small (at its maximum, it was usually between 10 and 20% of the voltage-independent component), the records of currents elicited were averaged over 10–30 times to improve the signal/noise ratio.

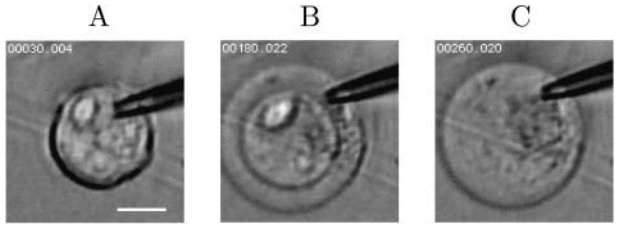

FIGURE 1 Injection of a trypsin-containing medium into a prestin-transfected cell. (*A*) A prestin-transfected cell before trypsin injection. The cell is in the whole-cell recording configuration. The scale bar is 10 μm. (*B*) The intracellular medium that contain 0.1 mg/ml trypsin is injected from the pipette (*upper right*) into the cell. The cell membrane with less optical density bulges out and then expands larger than the original size of the cell. (*C*) The same cell 3 min after trypsin injection. The cell membrane has low optical density and is uniform.

The membrane capacitance observed had bell-shaped dependence on the membrane potential, which was consistent with previous reports. Curve fit with the Boltzmann model (Eq. 1) indicated that the voltage that maximizes the capacitance (the peak voltage) was ~−40 mV. The steepness of the voltage dependence of the membrane capacitance indicated that the mobile charge $\hat{q}$ was between 0.7 *e* and 0.8 *e*, with *e* the electronic charge (Table 1). These values did not significantly differ from those obtained before trypsin treatment.

When the holding potential V_h was more negative than −82 mV, we could not keep tight seals for >5 min. If the holding potential was ~−80 mV, the peak voltage constantly shifted to more positive values. Application of pressure through the recording pipette accelerated the positive shift and removal of pressure simply slowed the shift. We found that holding potential of ~−78 mV was advantageous in maintaining the peak voltage at zero pressure relatively stable.

Application of positive pressure through the recording pipette shifted the peak voltage to a more positive value and its removal resulted in a negative shift of the peak voltage. Fig. 2 shows the data set with the largest pressure sensitivity. We determined a peak shift as a mean of voltage shift on application of pressure and the shift on removal of pipette pressure. The mean pressure sensitivity was (58.9 ± 4.5) mV/kPa (throughout this article uncertainties are expressed by standard deviations), $N = 6$. The voltage shift plotted against (1/2)*RP*, which corresponds to membrane tension according to Laplace's law (see Eq. 3), had mean slope of (10.3 ± 1.5) V·m/N. Here *R* is the radius of the cell and *P* is the pressure delivered to the cell through the recording pipette. The steepest slope that we obtained was (20 ± 4) V·m/N (Fig. 3 *A*). The values obtained at −80 mV holding potential were not larger. There was no correlation between these values and the ratio of the voltage-dependent component of the membrane capacitance, which ranged from 1 to 10 pF, to the voltage-independent component, which ranged from 15 to 40 pF.

Because GFP alone did not bring about a voltage-dependent component in the membrane capacitance with or without intracellular trypsin treatment, the voltage-dependent component was associated with prestin. Thus the voltage shift did not depend on the density of prestin in the membrane.

TABLE 1 Mobile charge $\hat{q}$ that determines the sharpness of voltage dependence before and after trypsin treatment

	Untreated	Trypsin-treated			
V_h	−75 mV	−75 mV		−78 mV	
P (kPa)	0	0	0.18	0	0.15
$\hat{q}$ (*e*)	0.72 ± 0.05 (7)	0.73 ± 0.06 (3)	0.75 ± 0.06 (3)	0.73 ± 0.06 (4)	0.74 ± 0.05 (4)

Pressure *P* is in kPa and $\hat{q}$ is in electronic charge *e*. Mean values and standard deviations are shown. V_h is the holding potential.

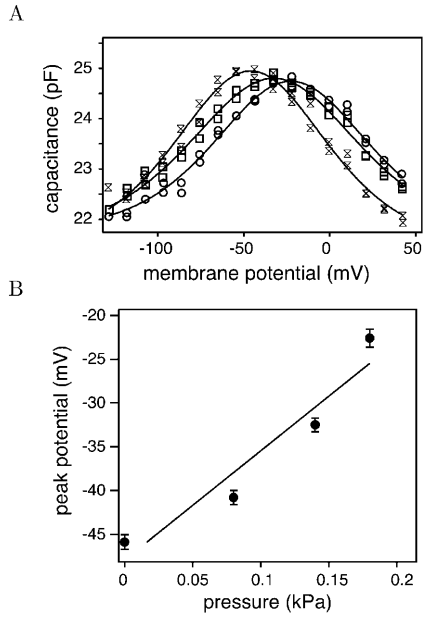

FIGURE 2 Shift in the capacitance peak due to applied pressure. The cell is trypsin treated. (*A*) Voltage dependence of the membrane capacitance. $P =$ 0 kPa (X); $P = 0.14$ kPa (□); and $P = 0.18$ kPa (○). The holding potential is −78 mV. Solid lines are curve fits using Eq. 1. Parameter values are $\hat{q}$= (0.89 ± 0.06)*e*, C_{lin} = (2.15 ± 0.2) pF, V_{pk} = (−45.9 ± 0.9) mV for $P =$ 0 kPa; $\hat{q}$= (0.80 ± 0.06)*e*, C_{lin} = (21.7 ± 0.2) pF, V_{pk} = (−32.5 ± 0.8) mV for $P = 0.14$ kPa; $\hat{q}$= (0.87 ± 0.06)*e*, C_{lin} = (21.7 ± 0.2) pF, and V_{pk} = (−22.6 ± 1.0) mV for $P = 0.18$ kPa. (*B*) Pressure dependence of the peak potential. Voltage dependence is shown in Fig. 2 *A* except for the data point at $P = 0.08$ kPa. The least square fit (*solid line*) has a slope (120 ± 15) mV/kPa.

Peak shift of not fully inflated cells

When trypsin-treated cells were under whole-cell voltage clamp with the holding potential more positive than ~−77 mV, the cell radius decreased but the voltage of capacitance peak for zero pressure ($P = 0$) was relatively stable. Pressure applied to the cell though the recording pipette shifted the capacitance peak but it did not have a significant effect on the cell radius, which continued to decrease. The pressure dependence was (25.8 ± 1.1) mV/kPa, $N = 6$. If the voltage shift is plotted against (1/2)*RP*, the slope is (6.1 ± 0.4) V·m/N (Fig. 3 *B*). These values are less than the values of those cells held at −78 mV.

Peak shift in untreated cells

As a control, we measured the pressure dependence of the membrane capacitance in untreated cells. When pressure was applied to the cell interior through the recording pipette, the peak-capacitance voltage shifted to a more positive value. The sensitivity was (11.3 ± 0.1) mV/kPa, $N = 5$. If we plot shift in the voltage peak against (1/2)*RP* to compensate for the possible effect of cell diameter, the peak shift is (3.9 ± 0.6) V·m/N (Fig. 3 *C*), less sensitive than trypsin-treated cells.

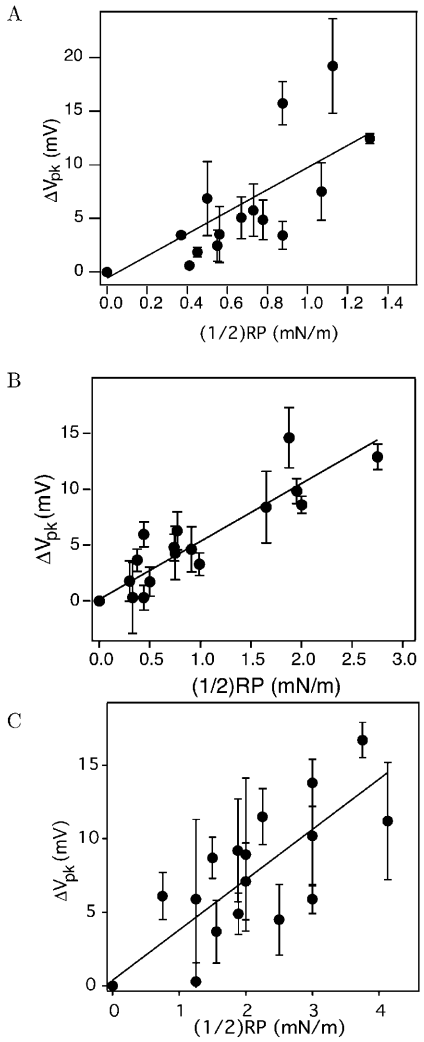

FIGURE 3 Voltage shift ΔV_{pk} plotted against (1/2)*RP*. (*A*) Trypsin-treated cells held at −78 mV. The slope of the least square fit (*solid line*) is (10.3 ± 1.5) V·m/N. (*B*) Trypsin-treated cells held at −75 mV. The slope of the least square fit (*solid line*) is (5.17 ± 0.40) V·m/N. (*C*) Untreated cells. The slope of the least square fit is (3.4 ± 0.3) V·m/N. Notice different scales in the abscissas in plots *A*, *B*, and *C*. The bars show standard deviations.

DISCUSSION

Membrane tension and mobile charge

When the cells were not treated with trypsin, their shape and radius are not sensitive to the holding potential. What is the mechanism for the sensitivity of trypsin-treated cells to the holding potential?

Apparently, their volume changes are related to ionic flow induced by the holding potential. Although it is reminiscent of volume changes in outer hair cells, which are dominated by potassium current (Iwasa, 1996), our system differs from those cells in two regards. First, K^+ is replaced by Cs^+, a K^+-channel blocker. Second, −77 mV, the holding potential critical for cell volume, is far from the resting potential of from ~−5 to −10 mV. Volume loss at the resting membrane potential indicates the presence of electrically silent efflux of electrolytes, possibly due to an ionic cotransporter or capillary effect. If we assume that the current across the cell membrane is carried by cations and the current between the pipette and the cell is carried by Cs^+ and Cl^-, imposing a membrane potential more positive than the reversal potential results in electrolyte loss and more negative potential leads to electrolyte gain (Iwasa, 1996). These two factors can balance at ~−77 mV. The gain or loss of electrolytes leads to a corresponding volume change if the cell is not mechanically constrained. This expectation is consistent with our observation.

In untreated cells the cytoskeleton can bear stress and reduces tension applied to the plasma membrane, resulting in reduced pressure sensitivity (Fig. 3 *C*). That appears to be the case also for a previous report (Santos-Sacchi et al., 2001). With the cytoskeleton digested, the plasma membrane is unable to resist volume changes. Thus, in trypsin-treated cells under voltage clamp, gain or loss of electrolytes due to ionic currents may determine cell volume.

When the cells are held at holding potential more negative than ~−77 mV, they are fully inflated, judged from the absence of radius changes. Because bringing the holding potential more negative away from the reversal potential increases current that introduces electrolytes into the cell, it is easy to understand that the whole-cell configuration cannot be maintained at a too negative holding potential as we have observed.

Thus, it is for trypsin-treated cells held at −78 mV that we may assume Laplace's law

$$T_m = (1/2)PR, \tag{3}$$

for estimating membrane tension T_m. Here P is applied pressure and R is the radius of the cell. Thus our value for prestin's tension sensitivity is (10.3 ± 1.5) V·m/N (Fig. 3 *C*).

When the holding potential is more positive than −77 mV, the cells are not fully inflated even though they maintain an approximately spherical shape. Certainly the cell's outline appears to be more rugged and contrasty as time passes under this condition. When positive pressure is applied to the cell from the recording pipette, the applied pressure is countered by an increase in membrane tension. However, the tension increase cannot be described by Laplace's law using the radius of the cell because local radii of membrane curvature must be smaller than the radius of the cell. The resulting deficit of force countering applied pressure must be balanced by the force due to electrolyte flow controlled by the holding potential.

For this reason, if Laplace's law is used for estimating membrane tension in this case, it should give an over-estimation of membrane tension. Indeed, the observed voltage shift at holding potential of −75 mV plotted against (1/2)*RP* has a slope less steep than the slope for cells held at −78 mV (Fig. 3).

Charge transfer $\hat{q}$ across the membrane by a prestin molecule is determined from Eq. 1, which describes the voltage dependence of the membrane capacitance together with phenomenological number $\hat{N}$ of prestin and the peak potential V_{pk}.

As in outer hair cells, trypsin treatment does not significantly change the charge $\hat{q}$ at zero pressure (Kakehata and Santos-Sacchi, 1995; Adachi and Iwasa, 1999). Application of positive pressure to the cell through the recording pipette shifts the peak potential V_{pk} to more positive values in both systems. However, unlike outer hair cells, the apparent charge $\hat{q}$ is not changed by applied pressure (Table 1). This observation can be understood as a result of lower density of prestin in transfected cells (see Appendix). For these reasons, values for phenomenological charge $\hat{q}$ obtained from Eq. 1 may give good estimates of charge transfer q across the membrane by prestin in cells whether pressure is applied or not.

Area changes of prestin

The voltage dependence of the membrane capacitance of transfected cells is due to prestin because nontransfected cells did not show such a voltage dependence. This voltage dependence is determined by the free energy difference F_{diff} of the two prestin conformations. Similar to the motor in outer hair cells, we may write (Adachi and Iwasa, 1999),

$$F_{diff} = q(V_m - V_{pk}) = F_0 + q \cdot V_m + a \cdot T_m. \quad (4)$$

Here V_m is the membrane potential, V_{pk} is the membrane potential that maximizes the capacitance. Quantities q and a are, respectively, the charge transferred by prestin and membrane area changes of prestin during conformational transitions. F_0 is a constant.

If a change ΔT_m in membrane tension induces the shift ΔV_{pk} in the peak potential, Eq. 4 leads to

$$a = q \cdot \Delta V_{pk}/\Delta T_m, \quad (5)$$

which gives the area difference a of prestin between its two states.

The sensitivity of the membrane capacitance to tension indicates that area changes of prestin associated with transfer of charge is (1.3 ± 0.2) nm^2 on the average (Fig. 4). One data set showed the largest value for area changes of (2.3 ± 0.4) nm^2.

Comparison with the motor in outer hair cells

Area changes of the membrane motor in outer hair cells have been determined under a condition similar to this study (Adachi and Iwasa, 1999). These outer hair cells are trypsin treated from inside and are made spherical by inflation. The mobile charge in these cells is ~0.8 e, similar to the value for prestin in transfected cells. However, the value for area changes of the motor in outer hair cells is ~4 nm^2, larger than the value (1.3 ± 0.1) nm^2 for prestin in transfected cells. The value for the outer hair cell motor is still larger than the maximum value (2.3 ± 0.4) nm^2 for prestin.

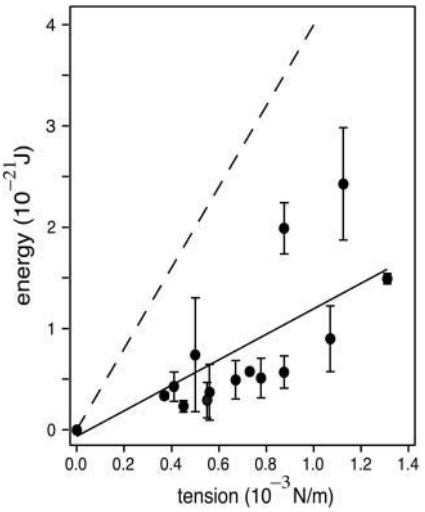

FIGURE 4 Area changes a of prestin can be determined by the slope when energy $\hat{q}\Delta V_{pk}$ is plotted against tension $(1/2)RP$. Data shown in Fig. 3 *A* is used for the estimation. The solid line shows the least square fit with a slope (1.3 ± 0.2) nm^2. The broken line shows the slope that corresponds to area changes of 4 nm^2. The vertical bars show standard deviations.

What is the reason for the difference? It can be explained by presence or absence of elements that interact with prestin in outer hair cells and/or in transfected cells. In the following, we examine specifically whether or not our data supports complex formation involving prestin in outer hair cells. A motivation for the examination is that the formation of a raft or cluster involving functional proteins is frequently observed in cell membranes (Brown and London, 1998). A specific motivation for examining complex formation of prestin is due to 10-nm particles in outer hair cells. The importance of 10-nm particles has been examined earlier. The localization of prestin (Belyantseva et al., 2000) agrees with that of 10-nm particles (Gulley and Reese, 1977; Kalinec et al., 1992; Santos-Sacchi et al., 1998). The difference in the density of 10 nm particles and the mobile charge (Santos-Sacchi et al., 1998) that is attributable to prestin suggests that each 10-nm particle contains about two prestin molecules. With 744 amino acids, the molecular weight of prestin is ~82 kD, which should take up 1.4×10^{-19} cm^3. If a 10-nm particle is approximated by a cube, its volume would be 10^{-18} cm^3. This implies that prestin may takes up only ~1/3 of the volume of a 10-nm particle, consistent with complex formation.

One other explanation for the observed difference in the magnitude of area changes is susceptibility to trypsin. Prestin could be degraded by trypsin in transfected cells whereas in outer hair cells it may be protected by associated proteins. However, we do not have evidence that prestin in transfected

cells is degraded. The mobile charge, $\hat{q}$, which corresponds to the sharpness of voltage dependence of transfected cells, did not show a noticeable difference before and after trypsin injection (Table 1). Even though the peak voltage at $P = 0$ showed a gradual positive shift of up to ~10 mV in ~10 min immediately after trypsin injection and a negative shift later, the tension sensitivity did not change with time. These observations are unfavorable to the interpretation that susceptibility to trypsin is responsible for the difference. However, we cannot completely rule out this interpretation.

Another explanation is that the formation of a complex involving prestin in outer hair cells enhances area changes. This explanation requires that prestin is insensitive to trypsin both in transfected cells and outer hair cells. In addition, membrane proteins that associate with prestin in outer hair cells are insensitive to trypsin. Such assumptions are indeed consistent with the observation that trypsin treatment does not reduce area changes of outer hair cell motor (Adachi and Iwasa, 1999). A mechanism in which a protein complex increases area changes could be an allosteric interaction between proteins that form a functional complex. Such an amplification of area changes needs to accompany a reduction in force generation.

Alternative models for motor activity

We have interpreted our results based on the model that the membrane motor in outer hair cells and prestin in transfected cells undergo conformational transitions that involve both charge transfer and membrane area changes. Such a model based on conformational transitions is in line with other functional membrane proteins such as ion channels and ion transporters. Moreover, it is consistent with a piezoelectric reciprocal relationship observed in outer hair cells (Dong et al., 2002). Some recent reports, however, regard prestin as a modifier that enhances motility based on surface charge (Zhang et al., 2001) or an electric dipole moment that twists the plasma membrane (Raphael et al., 2000). These interpretations were refuted earlier based on a number of experimental observations (Dallos and Fakler, 2002; Iwasa, 2001).

These results provide additional experimental evidence unfavorable to those interpretations. For example, the combination of trypsin treatment and inflation of prestin-transfected cells significantly changes the curvature of the plasma membrane. However, these treatments have little effect on the charge transfer due to prestin. These observations are inconsistent with these models that predict that motility and charge transfer are dependent on membrane curvature.

Other methods of applying known membrane tension

An alternative way of applying known membrane tension is to use the on-cell configuration of patch clamp. An advantage of on-cell configuration is that pressure can be applied across the membrane readily. A disadvantage is a technical difficulty in determining the curvature of sealed membrane patches. An additional problem is in an uncertainty involving undercoating of the plasma membrane in sealed membrane patches. Although cytoskeletal undercoating would be reduced during the formation of a tight seal, some component could remain (Sokabe et al., 1991). For these reasons, the on-cell configuration is convenient for confirming the existence of area changes (Ludwig et al., 2001). It is disadvantageous, however, in quantifying them.

The whole-cell recording configuration affords easy determination of the radius of membrane curvature. However, the effects of the cytoskeleton that reduces membrane tension need to be eliminated. Our method of removing this cytoskeleton is internal digestion by trypsin. Nonetheless it is hard to rule out possible effects of trypsin treatment on prestin or proteins associated with it.

CONCLUSIONS

We found that the sensitivity of prestin to membrane tension is (10.3 ± 1.5) V·m/N. This value indicates that the conformational transitions of prestin is accompanied by area changes of (1.3 ± 0.1) nm^2. Our observation confirms that prestin is indeed a motor protein based on piezoelectricity. The value for area changes is smaller than area changes of the motor in outer hair cells, which is ~4 nm^2. Consistent with previous reports, charge transfer of prestin across the membrane is about the same as that of the outer hair cell motor. The difference in the magnitude of area changes could suggest a prestin-containing complex, possibly a 10-nm particle in the lateral wall of outer hair cells.

APPENDIX: VOLTAGE DEPENDENCE

The mobile charge $\hat{q}$ that determines the sharpness of voltage dependence of the membrane capacitance is not changed by pressure applied to the cell in prestin-transfected cells (Table 1). In contrast, the capacitance peak in trypsin-treated outer hair cells undergoes an abrupt transition at applied pressure of 0.3 kPa, which is interpreted as the result of membrane area constraint due to cell inflation (Adachi and Iwasa, 1999).

What is the reason for the difference in these two systems? In the following we show that the difference can be explained mainly as the result of difference in the density of prestin.

In a two-state model of the membrane motor, the fraction f_ℓ of the extended state depends on the membrane potential V_m and membrane tension T_m,

$$\exp[-\beta(F_0 - qV_m - aT_m)] = \frac{f_\ell}{1 - f_\ell}, \qquad \text{(A1)}$$

where $\beta = 1/(k_B T)$ with the Boltzmann constant, T is the temperature, and F_0 is a constant. Quantities a and q are, respectively, the difference in the membrane area and charge transfer between the two states. The membrane charge Q transferable across the membrane due to conformational changes

of the motor is given by Nqf_ℓ, where N is the number of the motor in the cell membrane. Then the membrane capacitance due to the motor is dQ/dV_m. This relationship leads to

$$C_m = \beta a^2 N f_\ell (1 - f_\ell). \quad \text{(A2)}$$

Membrane area displacement S_{motor} due to the motor is given by aNf_ℓ. At very negative values of the membrane potential, f_ℓ is close to 1.

If the membrane is elastic, membrane tension is proportional to the area modulus K and elastic displacement $\Delta S_{elastic}$,

$$T_m = K \cdot \frac{\Delta S_{elastic}}{S_0}, \quad \text{(A3)}$$

where S_0 is the surface area of the spherical cell in a standard condition.

During short voltage pulses, the cell volume will be constant. If the cell is fully inflated, constant volume means constant surface area and thus a constraint of constant surface area is imposed during such an experiment. Thus the sum ΔS ($= \Delta S_{motor} + \Delta S_{elastic}$) is kept constant. At a very negative value of the membrane potential, $\Delta S \approx aN + \Delta S_{elastic}$. Under this condition, pressure P should not be completely determined by pipette pressure P_0, but it should be also affected by the surface area constraint. Thus, membrane tension T_m should be affected by pipette pressure P_0 as well as the state of the motor. However, such a reciprocal effect is not significant for prestin-transfected cells that we examined as we will see below.

We assume that membrane tension T_m is related to pressure difference P across the membrane by Laplace's equation, $T_m = 1/2\ RP$, where R is the radius of the cell. If we assume that pressure P_0 is applied to the cell while holding the membrane potential extremely negative, with the help of Eq. 3 we obtain

$$\frac{\Delta S}{S_0} = \frac{1}{2}\frac{RP_0}{K} + an, \quad \text{(A4)}$$

$$= \frac{T_m}{K} + anf_\ell, \quad \text{(A5)}$$

where $n = N/S_0$. This relationship leads to

$$T_m = \frac{1}{2}RP_0 - anK(1 - f_\ell), \quad \text{(A6)}$$

which shows that membrane tension T_m depends on the state of the motor because of area constraint.

Recall here that the fraction f_ℓ of the expanded conformation of the motor depends on T_m as described by Eq. 1. Thus f_ℓ is determined by the equation,

$$\exp[-\beta(F_0' - qV_m - \frac{1}{2}aRP_0 + a^2 nKf_\ell)] = \frac{f_\ell}{1 - f_\ell}, \quad \text{(A7)}$$

where F_0' is a constant.

Here we notice that the last term in the left-hand side of Eq. A7 provides negative feedback on f_ℓ, making transitions in the motor less sensitive to the membrane potential. This effect reduces the voltage dependence of the membrane capacitance in outer hair cells when they are fully inflated. The significance of this negative feedback is determined by the dimensionless factor $\beta a^2 nK$.

For outer hair cells, the density n is $\sim 7000/\mu m^2$, K is 0.13 N/m, and a is ~ 4 nm^2. Because $\beta = 1/(4.14 \times 10^{-18})$, this factor is ~ 4. This means negative feedback appreciably broadens conformational transitions. For transfected cells, prestin density n is $\sim 1/10$ of the density of the motor in outer hair cells, the area modulus is ~ 0.06 N/m, and a is ~ 2 nm^2. Thus the factor $a^2 nK$ is ~ 0.05. This means transitions of prestin are not significantly affected by negative feedback. This prediction is consistent with our present experimental observation.

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