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Electrokinetic properties of the mammalian tectorial membrane

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The tectorial membrane (TM) clearly plays a mechanical role in stimulating cochlear sensory receptors, but the presence of fixed charge in TM constituents suggests that electromechanical properties also may be important. Here, we measure the fixed charge density of the TM and show that this density of fixed charge is sufficient to affect mechanical properties and to generate electrokinetic motions. In particular, alternating currents applied to the middle and marginal zones of isolated TM segments evoke motions at audio frequencies (1–1,000 Hz). Electrically evoked motions are nanometer scaled (−5–900 nm), decrease with increasing stimulus frequency, and scale linearly over a broad range of electric field amplitudes (0.05–20 kV/m). These findings show that the mammalian TM is highly charged and suggest the importance of a unique TM electrokinetic mechanism.

Electrokinetic properties of the mammalian tectorial membrane

The mammalian cochlea is a remarkable sensor capable of detecting and analyzing sounds that generate subatomic vibrations (1). This extraordinary sensing property depends on mechanoelectrical transduction (MET) of cochlear hair cells (2, 3), which are functionally classified as inner and outer hair cells (OHCs). Both types of hair cells project stereociliary hair bundles from their apical surface toward an overlying extracellular matrix called the tectorial membrane (TM). Because of its strategic position above the hair bundles, the TM is believed to play a critical role in bundle deflection. Recently, genetic studies confirmed the importance of the TM in hair cell stimulation by highlighting how mutations of TM proteins cause severe hearing deficiencies (4, 5). In addition, mechanical strain produces electrical responses (e.g., streaming potentials (35)), and electrical stimulation may lead to mechanical deformations by a process referred to as electrokinesis (39, 40). The similarity between the structure and composition of the TM and those of other connective tissues suggests the possibility that charge might play a similarly important role in the function of the TM (27, 28).

In this study, we developed microfabricated devices that interface with the soft and fragile structure of fresh, excised TM segments to (i) measure the fixed charge density of the TM and (ii) apply oscillatory electric fields at audio frequencies to the TM. Using these methodologies, we demonstrate electrokinetic phenomena elicited by the presence of fixed charge and suggest implications for hearing mechanisms.

Results and Discussion

To determine the fixed charge concentration of the TM, we recorded the electrical responses of the TM using a microaperture chamber in a patch clamp configuration (Fig. 2; Materials and Methods). The voltage measured between the baths represents the sum of the junction potentials at two TM surfaces (Fig. 2A and B); one bathed in artificial endolymph (AE) and one bathed in a test solution. KCl concentration in the test solution was altered systematically to modulate the adjacent junction potential via changes in charge shielding. The resulting measurements were well fit by a model based on the Donnan relation (Materials and Methods) and provided an estimate of fixed charge density \(c_f\) of \(-7.1 \pm 2.0 \text{ mmol/L} \) at physiological pH, and 3.0 mmol/L at pH 3.5 (Fig. 2C). These results are consistent with predictions based on biochemical composition studies (27). The net charge of approximately \(-7.1 \text{ mmol/L} \) at physiological pH is large, representing 1 fixed charge molecule for every \(-25\) cations in endolymph.

To determine the mechanical contribution of charge in the TM, we analyzed a macrocontinuum model based on the stress–strain relation and Donnian equilibrium (27, 41). Internal pressures that

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is the hydraulic pressure, and $\pm C$ is absolute temperature, and $V_p$ is the volume in the absence of a hydraulic force.

Fig. 1. Electromechanical properties of the TM. Schematic drawing of the TM showing negative fixed-charge constituents attached to a network of mechanical springs (collagen fibers). In response to external pressure, the negative fixed charge and collagen fibers resist compression through electrostatic repulsion effects and mechanical forces, respectively.

arise from mechanical and electrostatic mechanisms resist external pressures on the TM (Fig. 1). The mechanical component is proportional to changes in TM volume and may be expressed as

$$P_{\text{mech}} = \frac{\kappa (V - V_0)}{V_0}, \quad [1]$$

where $P_{\text{mech}}$ is the hydraulic pressure, $\kappa$ is the bulk modulus, $V$ is TM volume, and $V_0$ is the volume in the absence of a hydraulic pressure. The electrostatic component depends on the concentration of fixed charge in the TM, which tends to concentrate mobile counterions to maintain electroneutrality. This concentration of counterions increases the osmotic pressure ($P_{\text{elec}}$) in the TM and may be expressed as

$$P_{\text{elec}} = RT \left( \left( \frac{C}{2} \right)^2 + C^2 - C \right). \quad [2]$$

where $R$ is the molar gas constant, $T$ is absolute temperature, and $C$ represents the concentration of monovalent ionic species (KCI) in the bath. This model predicts that electrostatic repulsion due to the $-7.1 \text{ mmol/L}$ of fixed charge density may contribute up to 0.35 kPa to the equilibrium bulk modulus $\kappa$, accounting for $\sim 70\%$ of compressive rigidity of the TM at equilibrium (21). Under dynamic conditions, the contribution of charge to mechanical properties also is important. A recent study found that a shift to acidic pHs in the bath causes about a two- to threefold reduction in dynamic shear impedance of the TM (42). The density of charge in the TM thus is sufficient to be the basis of mechanical properties under both static and dynamic conditions, a striking finding given the fact that the TM is highly hydrated (27, 29, 30).

The presence of charge in the TM suggests the possibility that electrical stimuli might generate a mechanical response (43, 44). The application of oscillating electric fields at audio frequencies (1–1,000 Hz) directed along the transverse axis of the TM in the microaperture chamber (Fig. 3) generated displacements of the TM near the marginal zone and Hensen’s stripe ($n = 4$ TM preparations) (Movie S1). Fig. 3B shows that TM displacements had peak amplitudes of 50 nm in response to 8 kV/m (at 10 Hz) at a position on the undersurface of the TM above the microaperture. Displacement amplitudes dropped significantly with distance away from the microaperture (Fig. 3B), increased with electric field strength (Fig. 3C), and decreased as a function of stimulus frequency with the phase angle approaching $-\pi/2$ radians (Fig. 3D), consistent with viscous dominated interactions. Similar motion behaviors were reproduced in a microfluidics chamber used to apply radial electric fields (SI Materials and Methods, Movie S2, and Fig. S1). Using this chamber, we measured the frequency response of the TM at very low frequencies (1–80 Hz) and exposed the TM to different perfusates in the microchannel environment. Perfusion of AE equilibrated at pH 3.5 led to a shift in phase angle of $-\pi$ radians (Fig. S2A), indicating ionization of positively charged collagen groups (38) and neutralization of negatively charged GAGs.

The frequency dependence of TM displacement magnitude and phase (Fig. 3D and Fig. S2B) suggests an interplay between electrohydrostatic forces on the solid matrix and electro-osmotic forces on interstitial fluid (which carries a charge equal in magnitude but opposite in polarity to the fixed charge on TM macromolecules) (40). At low frequencies, these forces displace the fixed-charge groups attached to the elastic matrix of the TM to generate displacements, which scale with field strength and the radius of the microaperture (45). However, this process is viscosity limited (Fig. 3D) because of the small size of the pores (21). Therefore, as the frequency of the electrical stimulus increases, there is less time per period for fluid motion through the porous matrix and a proportional reduction in total fluid transport. The result is that electrophoretic displacements of the TM are larger at low frequencies than at high frequencies.

**Fig. 2.** Fixed-charge density of the TM. (A) Schematic drawings of the microaperture setup showing side views of an isolated TM positioned over a circular microaperture. (Left) The microaperture creates a fluid path from the overlying bath of AE to the underlying microfluidics channel (test bath) perfused with AE-like solutions with variable KCl concentrations. (Right) The TM acts as an electrochemical barrier between the overlying bath and underlying fluid channel. The potential difference between the baths ($V_{AE} = V_f - V_c$) was recorded with Ag/AgCl electrodes that were placed in contact with the two baths. (B) Schematic drawing of the microaperture setup showing the top view of the TM positioned over the microaperture in the region near Hensen’s stripe and the marginal zone. (Inset) TM segment overlying the microaperture using 40x magnification. (C) Voltage was plotted as a function of test bath KCl concentration. Best-fit estimates to the median voltages yielded $\psi (-7.1 \pm 2.0 \text{ mmol/L})$, $n = 5$ TM preparations. Vertical lines and boxes denote extreme values and the extent of the interquartile range, respectively. Horizontal lines through the boxes denote the median values. Reducing the bath pH from 7.3 to 3.5 caused voltage measurements to change polarity and decrease in magnitude (3.0 mmol/L).
For asymptotically low frequencies, the solid and fluid phases separate, and electrokinetic displacements result from electrical forces acting on fixed charges embedded in the elastic matrix. To estimate the magnitude of these displacements, we modeled the TM as a semi-infinite, isotropic, elastic matrix, which overlies a circular aperture (25-μm diameter) through which current is passed. The mechanical properties of the matrix were represented by Young’s modulus (∼10^9 Pa for a basal TM segment) (18, 23). The matrix was assumed to have a uniform electrical resistivity (0.3 Ω m) and fixed charge density q (∼7 × 10^5 C/m^3), determined from the measurements in this study (c_f = 7 mmol/L; Fig. 2). The equations of motion were solved using finite differences (SI Results), with the result that electric fields of 1 kV/m (generated by ∼2 μA of current) produced electrokinetic displacements on the order of 1.5 μm. This motion estimate is based on a quasistatic model of the TM, which is appropriate at asymptotically low frequencies. However, the motion measurements in Fig. 3D show strong frequency dependence of motion (with slope of −1) over the measured range of frequencies (which is limited at low frequencies by table vibrations and thermal drift). We expect the trend shown in Fig. 3D to continue (to lower frequencies) until the transition to the transition to quasistatic behavior occurs, at frequencies that correspond to the poroelastic relaxation time [which is on the order of tens of minutes, based on previous osmotic experiments (21, 27)]. Thus, the transition frequency is two to three orders of magnitude below our lowest measured frequency, and we expect the quasistatic motion estimate to be two to three orders of magnitude larger than the measured range of motions.

Under dynamic conditions, viscous forces increasingly couple electro-osmotic and electrophoretic forces, which are equal in magnitude but opposite in direction. To test the effect of pore size and interstitial fluids on TM electrokinetics, we measured TM motion in response to changes in viscosity by introducing different-sized PEG molecules in AE (SI Results and Materials and Methods). We altered the viscosity of the fluid surrounding the TM by perfusing AE mixed with large PEG molecules (10 μmol/L,
400 kDa), which could not penetrate the TM. To alter internal viscosity of the TM, we perfused AE mixed with small PEG molecules (10 mmol/L, 8 kDa), whose radius of gyration was sufficiently small to enter through TM nanopores (21). Adding small PEG molecules caused TM motion to decrease by ∼6.5 times, whereas, adding large PEG molecules caused relatively minor changes in TM motion (Fig. 5C). Changing internal viscosity of the TM is functionally equivalent to reducing TM pore size, suggesting a strong dependence of TM electrokinetic interactions on effective porosity and a weak dependence on external viscosity of the surrounding fluid. These results provide a framework for a general poroelastic model of the TM under both quasistatic (21) and dynamic conditions, thereby highlighting the important interplay between fixed-charge groups, porosity, and the internal water content of the TM at the nanoscale.

Electrically evoked displacements of the TM may have important implications for electrical stimulation of the cochlea (46–50). Mechanical responses to electrical stimulation generally have been attributed to OHC somatic and hair bundle motility mechanisms. Although exogenous application of electrical currents undoubtedly excites sensory receptor cells, our results suggest that electrically induced TM motions also must be taken into account if electrical currents flow through the TM. Thus, electrically evoked motions of the cochlear partition may be stimulated at least partially by TM electrokinetics, especially at low audio frequencies.

Electrokinetic properties of the TM might interact directly with hair cell ion channels. Although the exact position of hair cell transduction channels relative to the TM remains unclear, it is well known that the undersurface of the TM is close (nanometer-scale separation) to the tallest rows of stereocilia (51–53). These electric fields, driven predominantly by potassium and calcium ions, generate electric fields, which in turn might exert local force on TM macromolecules. Although it is unlikely that transduction currents generate bulk movements of the TM in vivo, it is possible that local electrically induced deformations of the TM might occur near single stereocilia ion channels.

A previous study of electrical recordings from a bullfrog’s sacculus found electric field magnitudes of ∼1 V/m at distances ∼1 μm from the tips of the stereociliary hair bundles containing clusters of ion channels (51). These electric fields depend not only on the magnitude of the electric field but also on the spatial proximity of the TM relative to the ion channels. If we assume ∼20-pA transduction currents through a solitary ion channel (54, 55) and a distance of ∼1 μm from the channel opening, the electric fields would be in the range of those measured by Hudspeth (labeled “Ref. 51” in Fig. 4C). However, as the distance to the ion channel is decreased, the field strength increases rapidly. For example, at distances within 7–30 nm of an ion channel, the field strengths are 10^6–10^7 V/m, similar to those applied in the microaperture chamber (labeled “This study” in Fig. 4C).

Electrically evoked TM motions and forces produced by transduction electric fields depend on the material properties of the TM as well as the channel geometry. If we assume that a solitary ion channel is modeled as a single nanopore, then we can estimate the magnitude of TM displacements by scaling the measurements in the microaperture setup (Fig. 3). TM motion in the microaperture setup under quasistatic conditions (d_{ch} ∼1.5 μm) is proportional to the applied electric field strength (E_{ch} ∼10^3 V/m), where the proportionality constant is the effective charge (q_{ch}) divided by TM stiffness (k_{TM}). The stiffness k_{TM} scales with the radius of the microaperture (45) and therefore is ∼10^4 smaller near an ion channel (∼1-nm radius), whereas the quantity of charge q_{ch} scales with the volume of the TM overlying the microaperture. As a result, the proportionality between d_{ch} and E_{ch} changes by a factor of 10^7 for a single ion channel relative to the microaperture. On that basis, we predict TM displacements (d_{ch}) near an ion channel to be ∼0.01 nm in response to E_{ch} adjacent to a nanochannel (Fig. 4C). Because the electric field strength depends linearly on the number of stereociliary channels, the aggregate TM motion for ∼75 channels (n_{ch}) for a given hair bundle would be ∼0.75 nm. TM displacements at the ion channel and bundle level scale linearly with electrical current (I (SI Results)). This linear dependence of TM motion on electrical current is consistent with a finite difference model (SI Results), which predicts TM motion estimates on the order of those based purely on scaling.

The total mechanical energy available from TM electrokinetic motions (SI Results) is the product of d_{ch}^2 and TM stiffness (k_{TM} ≈ 1.33 N/m for a basal TM segment over a 5-μm radius) (18, 23, 45), yielding ∼0.75 aJ for basal TM segments and on the order of ∼7.5 aJ for apical TM segments with lower stiffness (k_{TM} ≈ 0.13 N/m for an apical TM segment over a 5-μm radius) (18, 23, 45). These energy estimates are comparable with the amount of work needed to deflect hair bundles during calcium-driven bundle
motility (~2 aJ) and are significantly larger than the work performed against viscosity during bundle movements (~0.1 aJ) (56, 57), suggesting that electrically evoked motions of the TM might interact with MET currents of the hair bundles. The direction of TM motion and the associated feedback mechanism at the level of the hair bundles depend on the location of the MET channels. If the ion channels face toward the inhibitory direction of bundle motion (i.e., if they are on the side closest to a shorter stereocilium), the net electric field caused by opening the channels will generate a force that will tend to close the channels, consistent with negative feedback. If, on the other hand, the ion channels face the tallest stereocilium (as in Fig. 4B), the net electric field would force TM negative charge groups in the direction of positive bundle deflection to open more ion channels, as the basis of a positive feedback mechanism for amplification.

Beyond cochlear mechanics, our findings have important implications for all sensory systems, which (with the exception of a few species of lizards) all contain accessory gelatinous structures overlying hair cells. Gels, such as the cupulae and otolithic membranes found in the vestibular system and those covering electroreceptive hair cells on the skin of aquatic vertebrates, play a key role in hair cell stimulation (58–60). Much like the TM, these gels are poised to undergo deformations in response to transduction currents. The effect of electrokinetics may be even greater in these sensory systems because their MET channels operate at significantly lower frequencies than those in the cochlea.

**Conclusions**

Results reported in this study show that the TM contains a high density of fixed charge that contributes to the compressive stiffness of the TM and may generate electrokinetic phenomena. Electrical stimuli applied to the TM evoke nanometer-scale displacements at audio frequencies by exerting force on the solid matrix (fixed-charge macromolecules) and fluid phase of the TM. This motion is directly related to the density of fixed charge inside the TM and may have important implications for cochlear mechanisms. Although the exact proximity of hair cell ion channels relative to the TM is not well known, the electric fields generated by MET are large near the undersurface of the TM and thus may generate local nanometer-scale deformations of the TM in vivo. Electrokinetic properties thus may play an important role in the deflection of cochlear hair bundles, suggesting, more broadly, that electrokinetic mechanisms may control the interaction of overlying gels and hair bundles in all sensory systems.

**Materials and Methods**

**Isolated TM Preparation.** TM segments were excised from the cochleae of adult male mice (strain CD-1, 4–8 wk old; Taconic) using a previously published surgical technique (61). The cochlea was surgically removed and placed in an AE bath containing 174 mM KCl, 5 mM Heps, 3 mM dextrose, 2 mM NaCl, and 0.02 mM CaCl₂. The bath was equilibrated at pH 7.3 at room temperature. The bone casing of the cochlea was chipped away gently until the TM from the cochlea. TM segments (typically 0.5–1 mm in length) were isolated from multiple animals in this study were approved by the Massachusetts Institute of Technology Committee on Animal Care.

**Microaperture Chamber.** The microaperture chamber contained a glass-drilled microaperture (12.5-μm radius) (Lenox Laser) connecting two fluid-filled compartments in the form of a microchannel and an overlying bath (Fig. 2A). The underlying microchannel was cast in a transparent elastomer called poly(dimethylsiloxane) (PDMS; Sylgard 184; Dow Corning) by applying a standard photolithography technique (62) in which a PDMS replica of the underlying channel was cured and subsequently sealed to a glass slide containing the microaperture. For fixed-charge density measurements, the top bath was perfused with AE and the microchannel was perfused with AE-like solutions in which the KCl concentration and pH were varied. KCl concentrations of 21 mM, 32 mM, 43 mM, 87 mM, and 174 mM were used at pH 7.3 and 3.5. Each test bath was perfused twice to test for repeatability. For electrokinetic measurements, the top bath and microchannel bath were perfused with AE.

**Measuring Fixed-Charge Density $c_f$**

Isolated TM segments were mounted in the microaperture chamber so that an electrochemical barrier separating the solutions in the two compartments was formed (Fig. 2A). The DC voltage difference ($V_D$) between the two compartments was measured with Ag/AgCl microelectrodes (A-M Systems) housed in large-tip micropipettes containing 3 M KCl solution and solidified in agarose. The electrodes were positioned in the top bath and in the reservoir connecting to the microchannel. This configuration allowed uniform electric fields to be delivered in the regions of the TM directly overlying the microaperture. The electrodes were coupled through an amplifier (DAM60-G Differential Amplifier, World Precision Instruments) and a multimeter (TX3 True RMS Multimeter, Tektronix) connected to a computer. DC potentials were stored in the computer at 2-s intervals. The DC potential difference between the two baths depends on the ionic concentration of the two baths and on TM fixed-charge density in this configuration. Therefore, by measuring the electric potential difference across the TM, we can estimate TM fixed-charge density. The potential difference arises primarily from Donnan potentials that form between each bath and the TM as a result of the presence of fixed charge. The potential between each bath and the TM is

$$ V = \frac{RT}{F} \left( \frac{c_1}{2C_{1f}} + \frac{c_2}{2C_{2f}} \right) + \ln \left( \frac{c_2}{c_1} \right) $$

where $V$ is the potential of the TM relative to the bath; $R$ is the molar gas constant; $T$ is absolute temperature; $F$ is Faraday's constant; $c_f$ is the concentration of fixed charge within the TM, which may be either a positive or negative quantity; and $C_f = (1/2c_f)$ is one half the sum of the concentrations $c_i$ of each ion in the bath. Because the bath consisted mainly of KCl, we estimated $C_f = (1/2)(c_1+c_2)$. If the two baths have identical compositions, the Donnan potential difference between the TM and each bath is identical, so the net potential difference between baths is zero. If one bath has a lower ionic concentration, the magnitude of the Donnan potential increases, so the potential difference between baths deviates from zero in a manner that depends on $c_f$ within the TM. When the two baths have dissimilar ionic strengths, in addition to the Donnan potential, a liquid junction potential (LJP) may arise between the baths. To measure the Donnan potential accurately, it is important to minimize and measure the LJP. In this respect, it is convenient that the TM normally resides in AE, which consists primarily of K⁺ and Cl⁻. Because these two ions have similar mobilities, the junction potential resulting from a concentration difference between baths is minimized.

**Measuring Electrokinetic Motion of the TM.** The microaperture setup also was used to apply voltages to TM segments. Isolated TM segments were placed over a microaperture with a radius of ~12.5 μm, and both compartments were perfused with AE. Sinusoidal voltages applied between the compartments generated electric fields in the central, middle and marginal zones of the TM overlying the aperture (Fig. 3 A and B). Displacements were visualized transversely across multiple focal planes within the bulk of the TM using computer vision and Doppler optical coherence microscopy (DOCM) techniques (18, 63).

We also developed a microfluidics chamber (Fig. 5I) used to apply electric fields to the TM in the radial (in-plane) direction (SI Materials and Methods) (44). Fluids were perfused through the microfluidics chamber, allowing equilibration of AE at different pHs and with PEG (Fig. S2 A and C) for TM motion amplitude, phase, and polarity studies.

**Motion Analysis with the Computer Vision System.** Images of the TM were collected optically with a 40x water immersion objective (0.8 N.A., Zeiss Axioplan) and a transmitted light condenser (0.8 N.A.). TM motion was measured with a 12-bit, 1,024 × 1,024-pixel CCD camera (C57-1024A; Dalsa Inc.) by strobing the light-emitting diode. The TM segment was illuminated at 16 evenly spaced stimulus cycles (over a stimulus cycle) at multiple optical planes separated by 1 μm. The collected images then were analyzed to determine the first eight harmonics of the periodic motion. We computed the magnitude and phase of radial displacement from the series of collected images by using previously published motion-tracking algorithms (18, 20).

**Motion Analysis with DOCM.** TM electrically evoked motions in the microaperture chamber were confirmed using a previously published DOCM system, which reconstructs motion from weakly scattering light reflected from multiple axial depths through the TM (63). The DOCM system has subnanometer resolution and was used to characterize TM motion as a function of stimulus frequencies.
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