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# Temperature dependence of non-linear capacitance in human embryonic kidney cells transfected with prestin, the outer hair cell motor protein

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**Abstract**

The transmembrane motor protein prestin is thought to underlie outer hair cell (OHC) motility. Prestin expressed in non-auditory cells confers OHC-like electrical characteristics to the cell membrane, including the generation of gating-like currents (or non-linear capacitance), whose voltage dependence is susceptible to membrane tension and initial voltage conditions. Here we report that prestin's voltage sensitivity is, like that of the native motor, markedly temperature dependent. Prestin-transfected HEK cells were whole-cell voltage clamped while temperature was varied from 10–35°C. $V_{pkcm}$, the voltage at peak capacitance, reversibly and linearly shifted to depolarized levels with increasing temperatures, while peak capacitance also increased, but with significant hysteresis upon recooling. Mathematical modeling suggests that this increase may be due to a charged voltage sensor having a wider range of movement through or larger unit charge within the plasma membrane at higher temperatures. 

*Keywords*: Prestin; Temperature; Capacitance; Cochlea; Membranes; Outer hair cell

The ability of outer hair cells (OHC) to generate mechanical responses to electrical stimulation is critical for the precise tonotopic tuning of the mammalian cochlea [2]. For several years it has been known that the mechanism underlying this motor activity is localized to the basolateral membrane of the OHC [5], and more recently a novel protein, prestin, has been identified as the putative motor protein [16]. Evidence for voltage-dependent mechanical activity of OHCs is easily detectable in whole-cell patch recording as a non-linear capacitance, or gating current, indicating the presence of a charged voltage-sensor participating in a conformational change [1,10]. Transfection of prestin into human embryonic kidney (HEK) cells confers non-linear capacitance with similar characteristics to that of the OHC lateral membrane[8,13,16]. Notably, transfected cells can exhibit electromotile responses in microchamber experiments [16], and excised patches from such cells have been shown to generate mechanical force in response to electrical stimulation [8]. These are necessary characteristics of the molecular mechanism for outer hair cell motility, and the finding that expression of prestin is necessary and sufficient to invoke them in non-auditory cells is convincing evidence that prestin is the motor protein of OHCs.

It remains in question, however, whether or not prestin alone is responsible for the mechanical properties of OHCs, or if an interaction with other molecular species in the OHC basolateral membrane is required. Evidence is accumulating that prestin alone can indeed account for all of the electromechanical properties of OHCs. One interesting aspect of OHC non-linear capacitance is that its voltage dependence is subject to modulation by such fundamental conditions as membrane tension [6], resting potential [10], and temperature [11]. Specifically, the voltage at which half maximal charge movement occurs, or, correspondingly, the voltage at peak capacitance ($V_{pkCm}$), can be shifted in the positive direction by increased intracellular pressure, negatively shifted holding potential, or increased temperature. The replication of these findings in prestin-transfected cells is crucial evidence that OHC electromotility relies on a monomolecular mechanism requiring only prestin. Effects of membrane tension [8,13] and holding potential [13] in prestin-transfected cells have been reported, and here we report on the effects of varying temperature. $V_{pkCm}$ in prestin-transfected cells is markedly dependent on temperature, in a manner no different from that of the OHC. Increasing

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temperature shifts $V_{pkCm}$ in a positive direction, and the effect appears to be linear in the temperature range under investigation. In addition, increasing temperature was seen to increase the magnitude of peak capacitance, but with significant hysteresis in the temperature dependence, suggesting that the relationship of peak capacitance to temperature is more complex than that of $V_{pkCm}$. Fitting of capacitance data to the first derivative of a two-state Boltzmann function [10] indicates that the temperature-dependent increase in capacitance is accounted for by an increase in the voltage-sensitivity of the OHC motor protein ($z$), but not an increase in the maximum charge moved ($Q_{max}$). These results suggest that movement of a voltage-sensor in the membrane is maximized at physiological temperature.

Transient transfection of HEK 293 cells was conducted as follows: Cells were cultured in MEME with 10% fetal calf serum. Cells were trypsinized and replated in 35 mm culture dishes 24 h prior to transfection with Lipofectamine 2000 (Life Technologies, MD). The transfection mixture consisted of OPTI-MEM media, Lipofectamine, the gPrestin-containing plasmid pcDNA3.1(−), and the GFP-containing plasmid pGreenLantern, used as a marker for successfully transfected cells. Generally, cells that successfully express one plasmid in a dual transfection will express both, and indeed, in the course of our recordings only one cell was found that exhibited green fluorescence (GFP) but not non-linear capacitance (prestin).

Twenty-four to 72 h after transfection, whole-cell patch clamp recordings were performed while varying bath temperature. The 35 mm culture dishes containing the cells were placed in a Peltier device (Dagan HCC-100A Heating/Cooling Bath Temperature Controller with HE-204 stage, Dagan Corp., MN). A temperature probe was inserted into the bath within 2 mm of the recorded cells. Cells were either patched at 10°C or cooled from room temperature after patching, after which the temperature was raised gradually up to 35°C and lowered back down to 10°C, and the cycle repeated as long as good recordings could be obtained from the cells. At 5-degree temperature intervals, we obtained full capacitance-voltage ($C$–$V$) functions as described below.

Round, isolated (non-coupled) cells exhibiting GFP-fluorescence were selected for electrophysiological recordings. Cells were whole-cell voltage clamped with an Axopatch 200A amplifier (Axon, CA) at a holding potential of 0 mV. The patch pipette solution contained (in mM): 130 CsCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES, pH 7.2. The external bath solution contained (in mM): 99.2 NaCl, 20 TEA-Cl, 20 CsCl, 2 $CoCl_2$, 1.47 $MgCl_2$, 10 HEPES, 2 $CaCl_2$, pH 7.2. Osmolarity was adjusted to 300 mOsm with glucose. Gigohm seals were obtained and electrode capacitance was compensated. Current responses were filtered at 10 kHz. Data collection and analyses were performed with a Windows-based whole-cell voltage clamp program, jClamp (SciSoft, CT, USA), using a National Instruments PCI-6052 interface.

The magnitude of non-linear membrane capacitance was evaluated using a continuous high-resolution (2.56 ms sampling) two-sine voltage stimulus protocol (10 mV peak at both 390.6 and 781.2 Hz), with subsequent FFT-based admittance analysis [12]. These high-frequency sinusoids were superimposed on a sinusoidal stimulus protocol sweeping from 0 to −175 mV, up to +175 mV, and back down to 0 mV. $C$–$V$ data were fit to the first derivative of a two-state Boltzmann function [10]where $Q_{max}$ is the maximum non-linear charge moved, $V_{pkcm}$ is voltage at peak capacitance or half maximal non-linear charge transfer, $V_m$ is membrane potential, $C_{lin}$ is linear capacitance, $z$ is valence, $e$ is electron charge, $k$ is Boltzmann's constant, and $T$ is absolute temperature. Gating currents were obtained with a standard P/-5 protocol, at a subtraction potential of +50 mV, as previously described [10]. In addition, in some experiments $V_{pkCm}$ was continuously tracked with 2 mV resolution by monitoring the reversal of gating current polarity as fully described previously [6]. This real-time tracking of $V_{pkCm}$ allowed a direct observation of the temperature dependence of non-linear capacitance, as $V_{pkCm}$ was observed to continuously follow changes in temperature in a reversible fashion.

Our results were very similar to those obtained from OHCs [11]. Fig. 1 plots non-linear capacitance vs. voltage for a cell at two temperatures. Two main effects of temperature can be seen - at the higher temperature, the magnitude of the capacitance is slightly greater, and the voltage at which the peak capacitance occurs is shifted to the right, to a more positive voltage. In all transfected cells exhibiting non-linear capacitance, $V_{pkCm}$ was seen to shift linearly in a positive direction with increasing temperature, at a rate of about 16.1 ± 6.8 mv/10°C ($n = 5$). This result agrees well with findings in OHC cells, which exhibited an average shift of 19.7 mV/10°C. Fig. 2a illustrates that these effects are continuous and fully reversible, using the continuous tracking of $V_{pkCm}$ as described above.

In order to calculate the average rate of shift in $V_{pkcm}$ in response to temperature changes from the data of five cells, it was necessary to verify that the effect is linear in the temperature range studied. Fig. 2b, *top panel* illustrates that this is the case. Cells that were continuously held through a wide temperature range yielded data that consistently fit a linear regression function of $V_{pkCm}$ vs. temperature ($r^2 \sim 0.93$). Peak $C_m$ was also seen to increase with temperature (Fig. 2b, *bottom panel*), but there is a considerable amount of hysteresis in the response, rather than the simple linear temperature dependence of $V_{pkCm.}$ With cooling, peak $C_m$ in all cells was relatively stable until temperatures well below physiological (<25°C) were reached.

The reason for this non-linearity is not entirely clear. As Fig. 2 illustrates, there was often a lag in the temperature dependent change in peak capacitance, compared with the change in $V_{pkCm}$, which precisely followed temperature changes. The lag ranged up to three minutes, as seen in Fig. 2a. Fig. 2b *bottom panel* shows that peak $C_m$ continued to increase for a short while after heating, even when the cell was cooling back down. Note that this lag cannot be

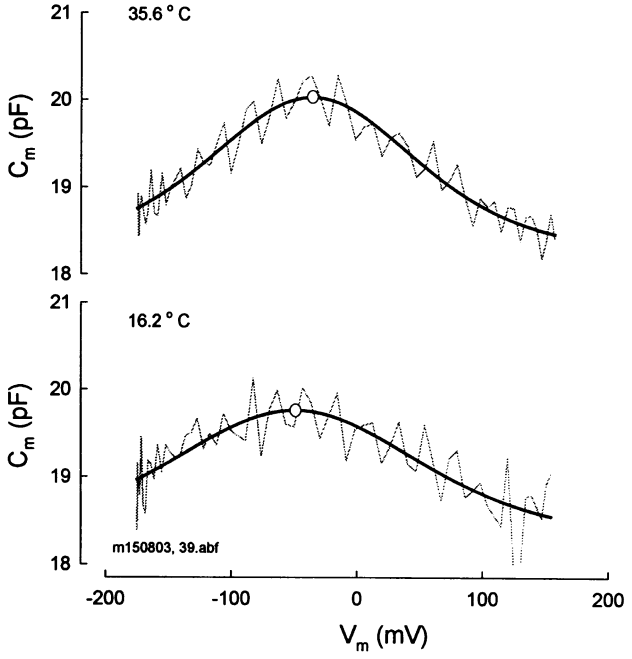


Fig. 1. Effect of temperature on non-linear capacitance of a prestin-transfected HEK cell. Capacitance was evaluated with a two-sine voltage stimulus protocol (see text) and the data were fit to the first derivative of a two-state Boltzmann function. Note two main effects of increasing temperature: a rightward (depolarizing) shift in the voltage at peak capacitance ($V_{pkCm}$), and an increase in peak capacitance. (35.6°C) $V_{pkCm}$: −32.2 mV, $z$: 0.49, $Q_{max}$: 0.37 pC, $C_{lin}$: 18.25 pF; (16.2°C) $V_{pkCm}$: −48.4 mV, $z$: 0.38, $Q_{max}$: 0.44 pC, $C_{lin}$: 18.17 pF.

explained by a discrepancy between the measured bath temperature and the actual temperature of the cell, since $V_{pkCm}$ does not show any such delay. Such slow effects may be due to changes in metabolic processes, or state of motor protein phosphorylation [3,4]. It is significant that sensitivity to temperature change was minimal at physiological temperatures. Our results are comparable to those of Khvoles et al. [7], who found that the amplitude of otoacoustic emissions from rat cochleae is maximal and nearly temperature insensitive at temperatures approaching physiological, but is significantly and reversibly depressed at higher and lower temperatures. Otoacoustic emissions are a byproduct of the so-called 'cochlear amplifier,' which is thought to be dependent on the mechanical responses of OHCs. In related studies, Seifert et al. [14,15] found that the amplitude of otoacoustic emissions in humans and guinea pigs declines with decreasing temperature, and recovers with considerable hysteresis. Detectable emissions tended to return while rewarming at lower temperatures than those at which they disappeared during cooling. The authors hypothesize that hypothermia causes a hearing loss associated with a decline in function of the cochlear amplifier, due to a reduction in OHC motility subsequent to a depression of the endocochlear potential (EP). However, our results suggest that the effect is direct, a decrease in prestin's non-linear capacitance being indicative of a decrease in its motor activity. Furthermore, the shift in the voltage-dependence (indicated by $V_{pkCm}$) of the cochlear amplifier would affect its gain and influence emissions, even if EP were to remain constant.

To investigate further the nature of the temperature-dependent changes in prestin's function, we fit voltage-dependent capacitance data to the first derivative of a two-state Boltzmann function, as described above and illustrated in Fig. 1. This fit allows for the derivation of the parameters $Q_{max,}$ the total amount of non-linear charge moved, and $z$, the effective charge movement of a single voltage-sensor. An increase of either of these parameters would result in the increased capacitance seen at higher temperatures. Fig. 3 shows $V_{pkCm}$, $Q_{max,}$ and $z$ plotted at two temperatures for each of five cells. There was not a reliable trend in the variation of $Q_{max,}$ which remains fairly stable, whereas $z$ shows a consistent increase with increasing temperature. Similar findings were previously reported in OHCs [11]. $z$ depends upon both the magnitude of charge and the distance it traverses within the membrane's electric field. Thus, the increase in $z$ may be caused by changes in either one of these factors. An increase in charge displacement may result from a decrease in plasma membrane viscosity at higher temperatures; the magnitude of charge may vary with phosphorylation and/or metabolism. Finally, it may be possible that some of the temperature-dependent effects that we observe could be due to a change in the availability of intracellular $Cl^-$, which is required for prestin activity [9]. These factors are expected to account for the non-linear effects of temperature on otoacoustic emissions [7,14,15], and their relative insensitivity to temperature changes in the physiological range. This insensitivity, reflecting properties of the motor protein prestin, would allow an animal to maintain

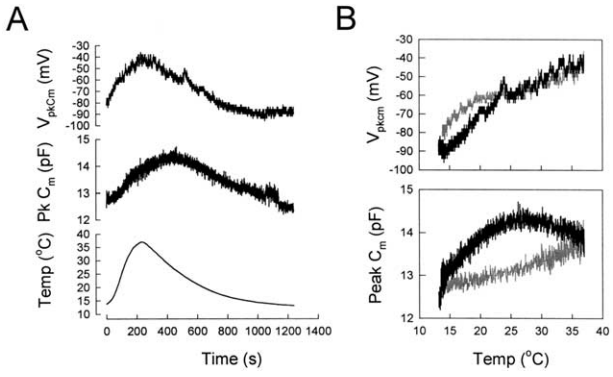


Fig. 2. (A) The voltage at peak capacitance ($V_{pkCm}$) and the peak capacitance ($C_m$) follow changes in temperature in a continuous and reversible manner. Here, a tracking procedure developed previously [6] was used to keep a cell voltage-clamped at $V_{pkCm}$ during heating and cooling. (B) $V_{pkcm}$ and peak $C_m$ plotted as a function of temperature for the same cell. Data from the heating phase is shown in gray, while data obtained during subsequent cooling is plotted in black. $V_{pkCm}$ increases nearly linearly with temperature (*top panel*), fitting a linear regression with $r^2 = 0.91$(heating), $r^2 = 0.95$ (cooling). However, $C_m$ shows considerable hysteresis in its response to changing temperature (*bottom panel*).

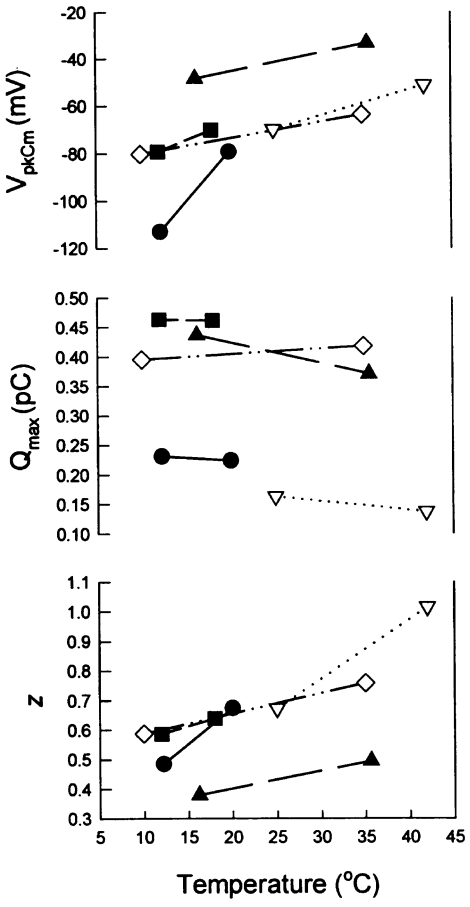


Fig. 3. The relationship between temperature and the parameters $V_{pkCm}$, $Q_{max}$, and $z$, derived from the fitting of capacitance data to a two-state Boltzmann function. Each of these quantities is plotted at two different temperatures for each of five cells. The increase in peak capacitance seen with increasing temperature cannot be due to an increased amount of total charge movement, because no clear increase is seen in $Q_{max}$. However, $z$ shows a clear increasing trend.

optimal cochlear function across small fluctuations in environmental temperature.

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