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On the temperature and tension dependence of the outer hair cell lateral membrane conductance G_{metL} and its relation to prestin

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Abstract Recently, we identified an outer hair cell (OHC) lateral membrane conductance, G_{metL}, that colocalizes with prestin and passes Cl^-, thereby influencing prestin's (SLC26A5) electromechanical activity. In this study, we report on a comparison of the temperature and tension dependence of G_{metL} and prestin. Though we find significant temperature and tension dependence of each, substantial differences exist which indicate their independent identity. The following data support this conclusion: (1) The voltage dependence of G_{metL} does not follow that of prestin's nonlinear capacitance (NLC) function when the latter is shifted by either temperature or membrane tension; (2) Unlike native OHCs whose NLC can be modulated by influx of extracellular Cl^-, prestin-transfected Chinese hamster ovary (CHO) cells do not show this phenomenon; (3) Stretch-sensitive, single channel currents are not evidenced after prestin transfection in CHO cells; and (4) There is no correlation between prestin expression level (gauged via NLC) and transmembrane current through G_{metL}. Thus, G_{metL} must result from the activity of another molecular species within the lateral membrane of the OHC. A clue to its identity is the conductance's nonlinear temperature dependence in contrast to prestin and other OHC conductances' linear dependence. Whereas K^+ conductances in OHCs present a uniform Q_{10} close to 1.2, G_{metL} shows a bimodal Q_{10}, with a Q_{10} of 1.5 below 34°C and a Q_{10} of greater than 4 and above. The dissociation of SLC26A5 (prestin) and G_{metL} theoretically provides for a modifiable anionic feedback to prestin via the degree of spatial separation between these interacting partners within the OHC lateral membrane.

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Introduction

The outer hair cell (OHC) lateral membrane motor, prestin or SLC26A5 [15, 27, 31], is thought to underlie the mammalian cochlear amplifier [14], and intracellular chloride is known to influence its activity [19, 21, 29]. This motor activity, measured either as a length change of the OHC or as an associated nonlinear capacitance (NLC), has been modeled successfully as a two-state Boltzmann process, with depolarization driving the motor into the compact state [10, 22, 23]. Recently, we discovered a stretch/voltage sensitive conductance (G_{metL}) that nonselectively passes small cations and anions through the OHC lateral membrane [21]. Furthermore, the intracellular accumulation of chloride via this conductance strongly influences prestin activity, increasing the probability of prestin being in the compact state. We found no indication of a conductance similar to G_{metL} in isolated neighboring Deiters' cells, which lack prestin.

G_{metL} shares some properties of prestin, namely, its restricted location within the lateral membrane, tension dependence, and modulation by Cl^- channel blockers [21]. In this regard, it is well established that some transporter family members actually underlie ionic conductances, including, for example, cystic fibrosis transmembrane conductance (CFTR) [8], the sodium/bicarbonate cotransporter, NBCn1 [4], and the glial glutamate transporter EAAT2 [20]. In fact, recently, another SLC26 family member, SLC26A7, has been shown to function as a chloride channel that is regulated by pH [13]. It is interesting to note, whereas SLC26A7 chloride currents are

blocked by DIDS, that G_{metL} is actually enhanced by DIDS [21], similar to the Na current associated with NBCn1 expression [4]. It is conceivable that G_{metL} could result from either the expression of prestin itself or through other proteins within the lateral membrane. In this study, we analyze the temperature and tension dependence of G_{metL} and prestin, thereby allowing us to determine whether prestin activity results in or influences Cl^- movements through membranes expressing this protein. Our data indicate that the molecular identity of G_{metL} is distinct from prestin, providing insight into their feedback capabilities.

Materials and methods

General

Guinea pigs were killed with halothane inhalation overdose in accordance with an approved protocol from Yale University's Animal Use and Care Committee. OHCs were freshly isolated from the adult guinea pig organ of Corti by sequential enzymatic (dispase 0.5 mg/mL) and mechanical treatment in Ca-free medium. Currents and capacitance from voltage-clamped cells were recorded at room temperature or at other temperatures (set by a Peltier device controlling the perfusion chamber) using an Axon 200A or 200B amplifier, Digidata 1321A or NI PCI-6052E board (Axon Inst., CA, USA; National Inst., USA), and the software program jClamp (Scisoft, CT, USA). To limit interfering K^+ conductances, the base intra- and extracellular solution was NaCl or TrisCl 140–150, $CaSO_4$ 2.5, $MgSO_4$ 1.2, HEPES 10, pH 7.2, 300 mOsm. Chloride substitutions were made with malate. For K^+ current studies, intracellular solutions were made with KCl. Solutions were delivered to individual cells by Y tube during continuous whole bath perfusion of base extracellular solution. Series resistance effects were corrected offline. Currents were monitored at the fixed holding potential stated in the figure legends. Evaluations of gerbil prestin were made in Chinese hamster ovary (CHO) cells. Transient cotransfection was achieved as described previously [16], with cotransfection of GFP. Data are presented as mean±SE.

Nonlinear capacitance

Nonlinear 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 fast Fourier transform-based admittance analysis as fully described in Santos-Sacchi et al. [24, 26]. These high-frequency sinusoids were superimposed on voltage ramps. To avoid capacitive current contamination (influenced by the cell's voltage-dependent capacitance) of I_{metL}, we used ramps of 1 s duration. Peak nonlinear capacitance was determined by subtracting linear capacitance. C–V data were fit with the first derivative of a two-state Boltzmann function and a constant representing the linear capacitance [22],

$$C_m = Q_{max}\frac{ze}{kT}\frac{b}{(1+b)^2} + C_{lin} \quad (1)$$

$$b = exp\left(\frac{-ze\left(V_m - V_{pkcm}\right)}{kT}\right)$$

where Q_{max} is the maximum nonlinear charge moved, V_{pkcm} is voltage at peak capacitance or half maximal nonlinear charge transfer, V_m is membrane potential, C_{lin} is linear capacitance, z is apparent valence, e is electron charge, k is Boltzmann's constant, and T is absolute

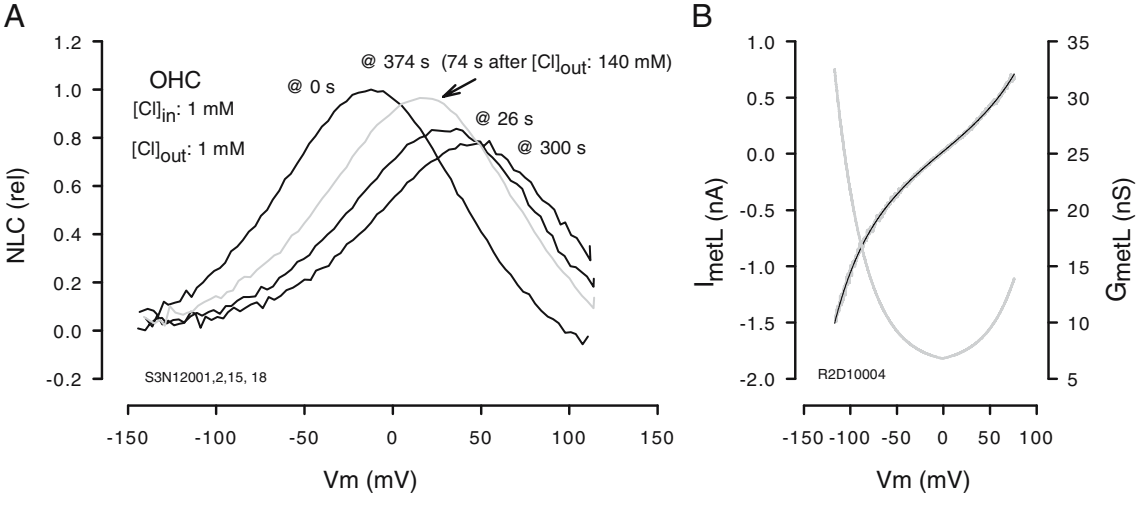


Fig. 1 Chloride enters the OHC through a lateral membrane conductance. **a** After obtaining whole-cell condition with 1 mM intra/extracellular Cl^- (Na-based) solutions, OHC NLC shifts rightward and decreases. NLC reaches steady state at about 2/3 of initial NLC beyond 1 min washout. Subsequently, a switch to 140 mM extracellular Cl^- restores NLC (*grey trace*), indicating Cl^- influx through G_{metL}. **b** Ionic current of an OHC with 150 mM Cl^- (Tris-based) intra/extracellular solution evoked by a 1 sec voltage ramp. The sigmoidal current is fitted with Eq. 2 (*solid line*; V_h: −0.71 mV) and the slope conductance (*grey*, *V-shaped line*) derived from the fit

temperature. A phenomenological equation was devised to fit the sigmoidal V_m dependence of I_{metL}, providing estimates of the midpoint voltage (V_h) of its operating voltage range, which were then compared with the midpoint voltage, V_{pkcm}, of the simultaneously recorded NLC.

$$I = A * dV * exp\left(|dV|/b\right) + off \quad (2)$$

where A is current amplitude, $dV=V_m-V_h$, b is a slope factor, and off is an offset.

Single channel recoding

Prestin-transfected CHO cells and controls were tested for the occurrence of stretch-activated single channel currents. Forty control CHO cells and 55 CHO cells transfected with normal prestin-yellow fluorescent protein were recorded by cell-attach configuration. Some of them were also recorded by inside-out configuration. Patches were made over fluorescent hot spots. Bath solution (mM): 140 NaCl, 2 $MgSO_4$, 2 $CaSO_4$, 10 HEPES; pipette solution (mM): 140 NaCl, 2 $MgSO_4$, 2 $CaSO_4$, 10 EGTA, 10 HEPES pH 7.2, 300 mOsm.

Results

NLC capacitance in the OHC is a shallow function of voltage (Fig. 1). The magnitude- and voltage-operating range is highly dependent on intracellular chloride levels but is also dependent in the native OHC on extracellular chloride which passes through a lateral membrane conductance. Figure 1a shows that, in the presence of low extracellular chloride (1 mM), washout of intracellular chloride with low chloride containing patch pipette solutions shifts NLC to depolarizing levels and decreases its peak magnitude. After reaching steady state, the introduction of normal extracellular chloride levels (140 mM) restores NLC. This restoration is a consequence of a gradient-driven, lateral membrane chloride current, I_{metL}, that has a V-shaped conductance, G_{metL}, and has been shown to be charge nonselective [21]. Fitting the slope conductance of the G_{metL} defines a midvoltage (V_h), where conductance is minimal, and establishes an operating voltage range which can be compared to that of NLC measured in the same cell. To determine whether the underlying molecular structure, prestin, which is responsible for NLC generation, also fosters G_{metL}, we simultaneously compared V_h and V_{pkcm}, under conditions where V_{pkcm} is known to change.

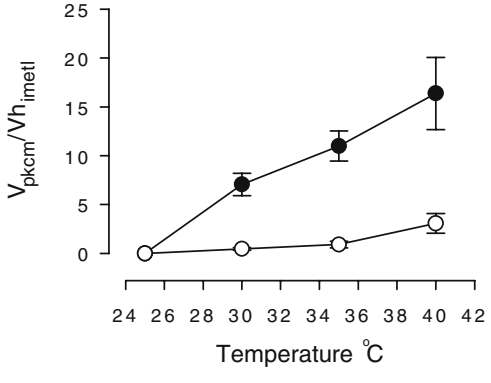


Fig. 2 After obtaining stable whole-cell conditions with 150 mM Cl^- (Tris-based) intra/extracellular solutions, bath temperature was altered. V_{pkcm} of the NLC (*filled circles*) shifted rightward (depolarizing) with increases in temperature; however *V*h of I_{metL} (*open circles*) did not follow, indicating independence of the two simultaneous measures. Plotted is mean±SE, n=4

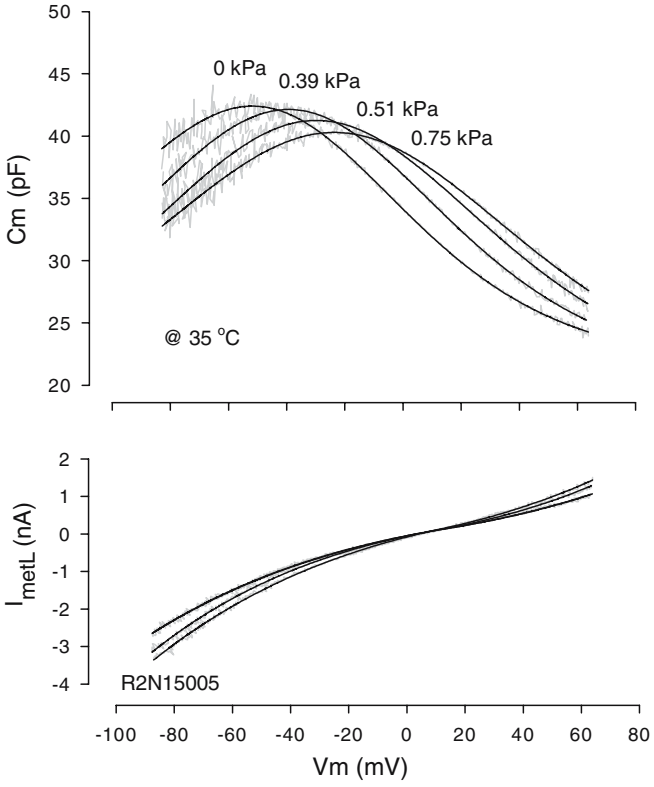


Fig. 3 After obtaining stable whole-cell conditions with 150 mM Cl^- (Tris-based) intra/extracellular solutions, tension was applied to the OHC membrane by pipette pressure. NLC shifts rightward and decreases with increasing turgor pressure. Note that I_{metL} increases with increasing membrane tension, but its voltage dependence remains unaltered. V_{pkcm} (mV) vs V_h (mV) with increasing pressure: −49.2/13.9; −39.0/14.2; −30.6/10.4; −23.0/11.3. Indeed, the initial turgor pressure change from 0 to 0.39 kPa produced a NLC shift, but no significant change in current, indicating the independence of the two simultaneous measures. Temperature was 35°C

First, we altered bath temperature (from 25 to 40°C), which has been shown to shift V_{pkcm} in the depolarizing direction [17, 25]. Figure 2 shows that whereas the operating voltage range of prestin is altered by temperature, shifting about 1 mV/°C, that of G_{metL} is little affected. Second, we changed intracellular turgor pressure to alter tension on the OHC membrane. As expected, increasing turgor pressure shifted V_{pkcm} to depolarizing levels [6, 9, 11, 27] (Fig. 3); however, the voltage-operating range of G_{metL} is unaffected. These observations were confirmed in more than five OHCs. Finally, we directly tested whether

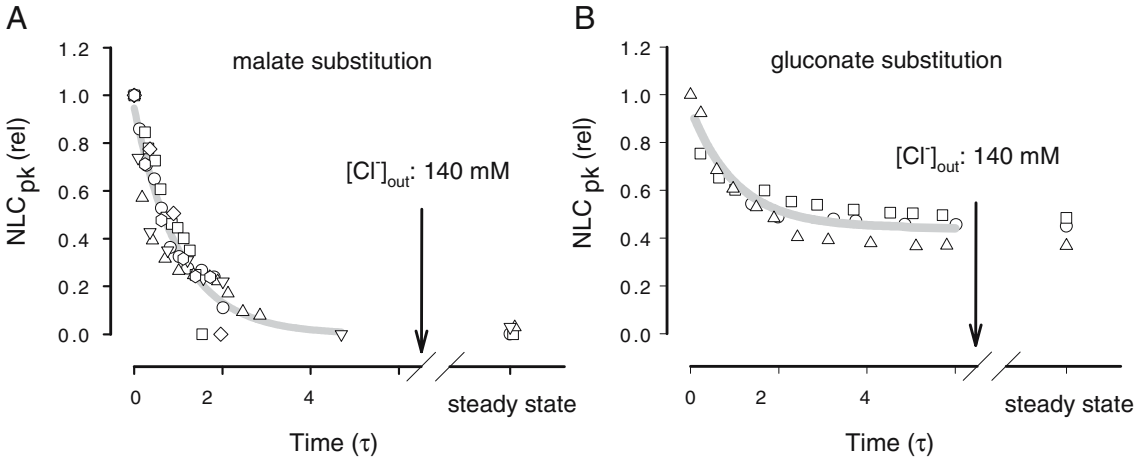


Fig. 4 **a**, **b** After obtaining whole cell in prestin-transfected cells with 1 mM intra/extracellular Cl^- (Na-based) solutions, NLC shifts rightward and decreases to barely perceptible levels when malate is the substitute anion. Using gluconate as substitute, reduction is only about 30%, similar to what we find in OHCs [21, 30]. Unlike in OHCs, a subsequent switch to 140 mM extracellular Cl^- does not restore NLC, indicating lack of Cl^- influx. *Symbols* denote individual cells whose washout effects were corrected for washout rate and plotted against the exponential washout time. The mean (±SE) value of τ was 49.18±7.57 s (malate); τ=35.56±10.00 s (gluconate)

prestin transfection in nonauditory cells results in the generation of a chloride conductance in addition to NLC. We previously showed that prestin targets the plasma membrane and generates NLC in CHO cells [18]. Now, we find that, as with OHCs in the presence of low extracellular chloride (1 mM), washout of intracellular chloride with low chloride containing patch pipette solutions shifts NLC to depolarizing levels and decreases its peak magnitude (Fig. 4). However, unlike native OHCs, the introduction of normal extracellular chloride levels (140 mM) fails to restore NLC. The degree of reduction in NLC during washout of chloride is dependent on the substitute anion. For malate, NLC decreases nearly fully (Fig. 4a); however, with gluconate as substitute, about 30% NLC remains (Fig. 4b), similar to what we find in OHCs [21, 29]. For either substitute anion, however, the subsequent reintroduction of high extracellular chloride does not increase NLC. Similar experiments carried out with gramicidin patches to eliminate pipette washout of chloride near the inner aspect of prestin gave the same results (Fig. 5a). On the other hand, when we increased intracellular chloride via the patch pipette after gramicidin patch rupture or when we

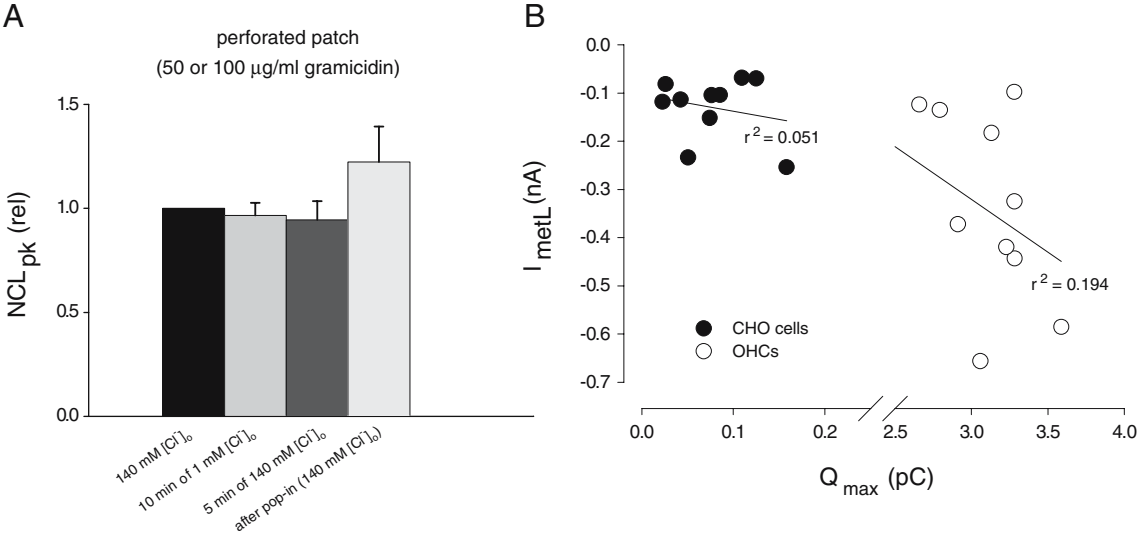
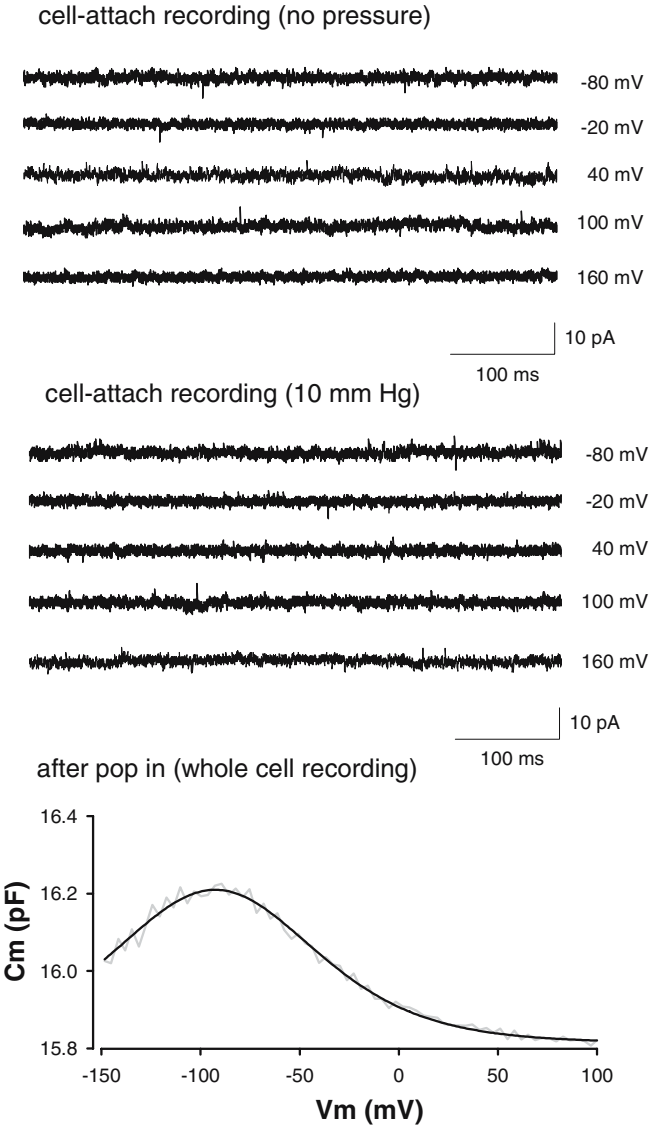


Fig. 5 **a** Lack of chloride influx in prestin-transfected CHO cells under perforated patch conditions. Prestin-transfected CHO cells were patched with gramicidin pipettes to limit the pipette washout of chloride so that transmembrane chloride flux could be unambiguously determined. In the presence of 140 mM extracellular chloride, relative NLC was determined after series resistance allowed whole-cell measurements. Subsequently, extracellular chloride was altered to see if NLC could be modulated by influx or outflux of chloride. Reductions and reperfusion of chloride had no effect, but NLC was augmented when true whole-cell configuration was established after patch rupture, indicating that chloride did not enter through the membrane. n=6 cells. **b** Comparison of G_{metL} and prestin activities shows no correlation in either prestin-transfected CHO cells or OHCs, as might have been expected if the expression level of prestin influenced I_{metL} activity. *Symbols* denote separate cells



◀ **Fig. 6** On cell patch recording of prestin-transfected CHO cells to determine single channel currents [before (*top panel*) and after (*middle panel*) stretch to patch]. In the prestin-transfected cell, shown stretch did not elicit single channel currents (*top and middle panels*). The *bottom panel* NLC was confirmed after patch rupture. Forty cells of control CHO cells and 55 transfected cells of prestin-YFP fusions were recorded by cell-attach configuration. Some of them were also recorded by inside-out configuration. No stretch-sensitive activity was found from the prestin group. In both groups, some non-stretch-activated endogenous currents were recorded. They were activated only at hyperpolarization potentials, i.e., around rest potential (−80 to −60 mV). The numbers are 7 out of 40 from control CHO cells and 5 out of 55 from prestin cells

reintroduced chloride via whole-cell pipette backfill after intracellular washout, NLC increased or recovered, respectively. We also compared prestin activity (Q_{max}) to G_{metL} activity (I_{metL} at −70 mV in symmetrical 140 Cl solutions) to see if there was a correlation between the two (Fig. 5b). In either CHO cells or OHCs, the activities were uncorrelated. In addition, we searched for stretch-dependent, single channel activity that might have arisen following prestin expression in CHO cells and found none (Fig. 6). Taken together, our data show that prestin is not responsible for the chloride conductance, G_{metL}, found in OHCs.

Finally, though we found no change in the voltage-operating range of G_{metL} with temperature (Fig. 2), I_{metL} magnitudes at −70 mV were bimodally temperature sensitive. Figure 7 shows that below about 34°C, the Q_{10} value was 1.58 (open circles, inset), but above that temperature, Q_{10} was 4.22 (closed circles). In comparison, in the absence of ionic-blocking solutions, K^+ currents at +30 mV (steady holding level) in OHCs (triangles) show Q_{10}s of less than 1.3.

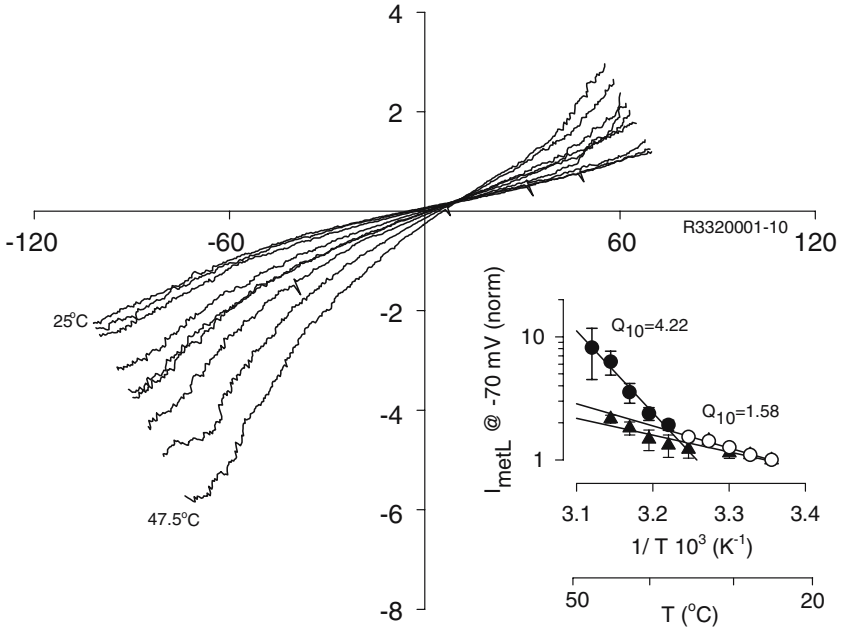


Fig. 7 I_{metL} is bimodally temperature sensitive, showing high sensitivity above 34°C, and typical diffusional sensitivity below that. K^+ current magnitude in OHCs is not temperature sensitive. OHC *I*metL at −70 mV, *circles*; OHC *I* K^+ +30 mV, *triangles*. Plotted is mean±SE, *n*=3–5. Series resistance correction was performed on a point by point basis ($V_m = V_{command} - I^*R_s$). The progressive limited range of voltages as a function of current magnitude results in the "tilted" appearance of the *I–V* curves

Discussion

G_{metL} is partially blocked by a few stretch-channel blockers, including gadolinium, tamoxifen, and quinine but is resistant to a host of other channel and transporter blockers [21]. Although the molecular identity of G_{metL} remains unknown, it is clear that G_{metL} and prestin share some characteristics, including restriction to the lateral membrane and sensitivity to chloride channel blockers (e.g., niflumic acid). One possibility is that G_{metL} arises from prestin itself, just as SLC26A7 underlies a chloride conductance [13]. Here, because we hypothesized that G_{metL} could arise from the conformational activity of prestin, we surmised that modulating prestin's voltage dependence with temperature and pressure would necessarily affect G_{metL}'s voltage dependence if the foregoing premise were true. To test whether prestin produces the OHC conductance or whether prestin somehow gates this conductance (possibly via a prestin-induced mechanical activation), we monitored characteristics of G_{metL} while modulating NLC with established techniques, including manipulations of turgor pressure and temperature, to see if each is similarly affected. In addition, we determined whether transfection of prestin into nonauditory cells induces a chloride conductance. The absence of a correspondence between the voltage-operating range of NLC and of G_{metL} in OHCs and an absence of significant Cl^- flux in prestin-transfected cells lead us to conclude that each is independent. This conclusion is further established by the absence of a correlation between Q_{max} and I_{metL} magnitudes in CHO cells and OHCs, and the absence of induced, stretch-dependent single channel activity in prestin-transfected CHO cells.

Another indication of the distinct nature of the two lateral membrane components is the temperature effects we observe on each. Though each is temperature sensitive, a clear breakpoint in sensitivity is evidenced for G_{metL}, where the conductance markedly changes its behavior as a characteristic temperature is crossed; NLC, on the other hand, is monotonically responsive to temperature, and this additionally indicates that possible phase transitions within the lateral membrane do not occur. That is, temperature has its effects on the protein structures underlying NLC and G_{metL}, not the membrane environment. This is not to say that temperature cannot affect prestin via the lipid bilayer, as we have shown that lipid reactive agents and, indeed, temperature can alter the kinetics of voltage- (prepulse) induced shifts in the motor's Boltzmann function along the voltage axis [28].

It is of interest that the temperature dependence of G_{metL} points to a molecular structure whose conformation is switched at a threshold temperature. Again, the absence of such a threshold phenomenon in the K^+ currents of OHCs argues against a nonspecific influence on the membrane. This type of behavior is reminiscent of some temperature sensitive transient receptor potential (TRP) channels [7], where Q_{10} above threshold temperatures, ranging from 30 to 50°C, can switch to greater than 10. It is also interesting that TRP channels can be mechanically sensitive, as is G_{metL}. Nevertheless, TRP channels are cation selective, and we have previously tested the TRP channel blocker, ruthenium red, without effect [21]. It is interesting that GLUT5 is known to be associated with prestin within the lateral membrane [1]; however, in our preliminary tests, cotransfection of GLUT5 and prestin into CHO cells did not generate chloride fluxes. It may be that I_{metL} may arise from other components within the lateral membrane, including other SLC26 family members whose residence has not yet been determined in the OHC. CFTR has also been found to interact with prestin in OHCs [30], though we have not found electrophysiologic evidence for this or other chloride channels in the OHC [21].

Currently, there are two mechanisms that may contribute to cochlear amplification in mammals. One mechanism involves stereociliary bundle mechanics, driven by ionic flux of Ca^{++} through the, as yet, molecularly unidentified {however, see [5] for a review} stereociliary mechanoelectrical transducer (MET) conductance [3, 12]. The other mechanism involves prestin-based electromotility of OHCs [2, 14, 31], which, being anion dependent [19], can be modulated by ionic flux of chloride through the, as yet, molecularly unidentified lateral membrane conductance, the G_{metL} [21, 30]. Our observations indicate that for the latter mechanism, interaction between ion and motor function arises from distinct molecular contributors, namely, prestin and G_{metL}, whereas in the former case, the MET conductance works on itself. This difference between feedback mechanisms may be important in understanding capabilities of each mechanism towards amplification. For example, whereas the molecular colocalization of the stereociliary bundle's feedback mechanism constrains timing of interactions between Ca^{++} and the channel, the distinct identities of prestin and G_{metL} could permit variable interaction delays simply by varying the distances between each within the lateral membrane. It is well known that setting appropriate feedback delays is crucial in nonlinear system performance, and, thus, having a potential means to adjust feedback delays may be viewed as particularly advantageous.

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