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Protein- and Lipid-Reactive Agents Alter Outer Hair Cell Lateral Membrane Motor Charge Movement

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Abstract. Outer hair cells from the mamma*lian cochlea are mechanically active cells that rely on charged voltage sensors within their lateral plasma membrane to gate the integral membrane motor protein, prestin, into one of two area states. Here we use protein and lipid reactive reagents to probe the influence of these bilayer components on motor-induced nonlinear membrane capacitance. Of the protein-reactive reagents tested, cross-linking and sulfhydryl reagents were most effective in altering steady state and time-varying motor activity. Of the lipid-altering agents, chloroform and HePC were most effective. Chloroform, in particular, drastically modified the susceptibility of the motor to prior voltage (initial conditions). Our data suggest that outer hair cell motor activity derives substantially from interactions with its lipid environment.

Key words: Motility — Gating charge — Membrane capacitance — Protein — Lipid

Introduction

Mechanical activity of the outer hair cell (OHC) underlies the enhanced sensitivity and frequency-resolving power of the mammalian cochlea (Dallos, 1992). The molecular motors that drive this activity harness electrical energy in a manner similar to other integral membrane proteins that generate gating currents upon voltage-induced conformational change (Armstrong & Bezanilla, 1974; Hilgemann, Nicoll & Philipson, 1991; Alkon, Etcheberrigaray, & Rojas, 1993, Bezanilla, 2002), namely, a motor-related displacement current or equivalently, a nonlinear capacitance (NLC) has been identified within the OHC's lateral membrane, which reflects conformational fluctuations of the motor (Ashmore, 1990; Santos-Sacchi, 1991; Huang & Santos-Sacchi, 1993; Wu & Santos-Sacchi 1998). Recently, Dallos and colleagues have identified the OHC motor protein as prestin, a member of the SLC26 transporter family (Zheng et al., 2000). Thus far, when expressed in non-auditory cells, this integral membrane protein displays all the known biophysical characteristics of the OHC lateral membrane motor (Zheng et al., 2000; Ludwig et al., 2001; Meltzer & Santos-Sacchi, 2001; Santos-Sacchi et al., 2001; Santos-Sacchi & Navarrete, 2002).

Integral membrane proteins, such as ionic channels and transporters, are influenced by their lipid bilayer environment (Goulian et al., 1998). Predictably, then, both protein-and lipid-reactive reagents would be capable of modulating prestin's function. We report here on the modulation of OHC NLC, including the effects of prior voltage on OHC NLC (Santos-Sacchi, Kakehata & Takahashi, 1998), by such agents and find that OHC motor protein functional characteristics likely arise from interactions with the lipid environment. Preliminary accounts of some portions of this work have been presented in abstract form (Santos-Sacchi & Wu, 1998).

Materials and Methods

Cell Preparation

Guinea pigs were anesthetized with halothane and killed by cervical dislocation. OHCs and supporting cells were isolated enzymatically with dispase I (0.5mg/ml for 10 min followed by gentle trituration through a polyethylene pipette) in a modified Leibovitz medium that contained (in mM): NaCl 142, KCl 5.37, $MgCl_2$ 1.47, HEPES 5, $CaCl_2$ 2 and dextrose 5; 300 mOsm, pH7.2. The cells were then transferred to a 700-μl perfusion chamber. Most experiments were conducted at room temperature (~23°C). Temperature for a subset of experiments was set by a Peltier device. A

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Nikon Diaphot inverted microscope with Hoffmann optics was used to observe the cells during electrical recording.

Voltage Clamp

Single OHCs were studied under whole-cell voltage-clamp conditions using an Axon 200A amplifier at a holding potential of −80mV. Initial resistances of patch pipettes were 2–3 MΩ, corresponding to tip sizes of 1–2 μm. Residual series resistance ranged from 3 to 7 MΩ. All data collection and analysis were performed with a Windows-based program, jClamp (SciSoft, CT). Ionic blocking solutions were used to remove voltage-dependent ionic conductances so that capacitive currents could be analyzed in isolation (Santos-Sacchi, 1991; Huang & Santos-Sacchi, 1993). The patch-pipette solution contained (in mM): CsCl 140, $MgCl_2$ 2, EGTA 10, HEPES 5, with pH 7.2 and osmolarity 300 mOsm (adjusted with dextrose). The external ionic blocking solution contained (in mM): $BaCl_2$ 10, $CoCl_2$ 2, $MgCl_2$ 1.47, NaCl 100, $CaCl_2$ 2, HEPES 5, with pH 7.2 and osmolarity 300 mOsm (adjusted with dextrose). Whole-chamber perfusion was continuous. Protein- and lipid-reactive reagents were perfused via a Y-tube directly onto the cell under study for short periods of time (1.5–10 min).

Reagents

Several protein-reactive reagents were tested, including glutaraldehyde (0.3%), N-ethylmaleimide (NEM; 2 mM), p-chloromercuriphenylsulfonate (pCMPS; 2 mM), dithiolthreitol (DTT; 2 mM) and diamide (2–5 mM). Glutaraldehyde is a dialdehyde fixative that works by crosslinking peptide chains; NEM, pCMPS and diamide are sulfhydryl reagents that react with cysteine groups; DTT is a reducing agent that breaks disulfide bonds [(Stirling,1975); *see* also Lundblad (1995) and references therein]. Lipid-reactive reagents included saturated chloroform, hexadecylphosphocholine (HePC; 2–10 mM), filipin (0.75 mg/ml), phospholipase A2 (PLA2; 1ng/ml), and triton (0.01%). Triton is a commonly used laboratory detergent. Chloroform is a commonly used lipid solvent. Filipin binds to cholesterol in membranes (Stetson & Wade, 1983). PLA2 cleaves fatty acyl chains from phospholipids (Danthi, Enyeart & Enyeart, 2003). HePC is a lysophospholipid analogue that can intercalate within plasma membrane (Rakotomanga, Loiseau & Saint-Pierre-Chazalet, 2004). The effects of these agents on the membrane likely ranges from altering its viscosity to changing its curvature and thickness, with subsequent effects on integral proteins.

Capacitance Measurements

Continuous high-resolution capacitance measures were acquired through admittance (Y) analysis at time resolutions of 5.12 or 2.56 ms, utilizing a two-sine-wave voltage-stimulus protocol (Santos-Sacchi et at., 1998). The stimulus consisted of the sum of two voltage sine waves (390.625 and 781.25 Hz), each at a magnitude (V) of 10 mV peak. This AC stimulus was superimposed on voltage-ramp or step stimuli. Capacitance-voltage functions were fit to the first derivative of a two-state Boltzmann function relating nonlinear charge to membrane voltage (dQ/dV; (Santos-Sacchi, 199l; Huang & Santos-Sacchi, 1993))

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

Where

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

$Q_{\max}$ is the maximum nonlinear charge moved, V_{pkCm} is voltage at peak capacitance or equivalently, at half-maximal nonlinear charge transfer, V_{m} is membrane potential, z is valence, C_{lin} is linear membrane capacitance, e is electron charge, k is Boltzmann's constant, and T is absolute temperature. All results are reported as mean ± SE. Gating currents were measured with the P/-5 technique at a subtraction holding potential of +50 mV (Armstrong & Bezanilla, 1977).

Pipette pressure was monitored via a T-connector to a pressure monitor (WPI, Sarasota, FL), and modified when required with a syringe connected to the Teflon tubing attached to the patch-pipette holder (Kakehata & Santos-Sacchi, 1995). The cylindrical shape of OHCs was maintained with slight positive pressure during data collection.

There are two potential, non-specific problems that must be considered when treating cell membranes with reactive agents. The membrane resistance (leakage) and/or the linear capacitance (e.g., membrane thickness) might be altered. With our dual-sine measurement technique, however, capacitance measures are resistant to membrane resistance changes. Also, linear as well as nonlinear capacitance are simultaneously measured. Changes in linear capacitance will simply shift the baseline of the NLC function, and should not interfere with the Boltzmann fit. Other than correcting steady-state voltages for series-resistance errors, which we do, we cannot correct for space-clamp changes. Nevertheless, estimates of delayed electrotonic spread in the OHC, about 70 μs for a cell of 70 μm (Ashmore 1986), indicate that our measures, which use frequencies below 800 Hz, should be reliable. Additionally, since the voltage-dependent conductances in the OHC are located in the basal pole of the cell (Santos-Sacchi, Huang & Wu 1997), the voltage drop along the lateral membrane where the motors are should be close to isopotential.

Results

Protein-reactive reagents

OHCs present a voltage-dependent C_{m} that arises from restricted charge movement within the lateral plasma membrane and this electrical measure of motor activity is modifiable by protein-reactive reagents (Fig. 1*A, B*). Low concentrations of the cross-linking fixative, glutaraldehyde (0.3%), caused a reduction in charge movement that probably indicates an abridged conformational change of the motor protein. Simultaneously, in all tested cells ($n = 5$) V_{pkcm} shifted in the hyperpolarizing direction. The effects were cumulative over the perfusion period (10 min in this case), indicating progressive inactivation of motors. In the control condition, prior to glutaraldehyde treatment, dc steps in voltage resulted in an immediate change in OHC C_{m} (Fig.1*C*, *top trace*), followed by an exponential decay that obtains from a voltage- and time-dependent shift in the voltage operating range (i.e., V_{pkcm}) of motor charge movement. This phenomenon is referred to hereafter as an amplificatory shift, since at a fixed voltage it represents a time-dependent recruitment of motors into the same conformational state evoked by the eliciting voltage step (Santos-Sacchi et al., 1998). Glutaraldehyde treatment prevented this time-dependent change

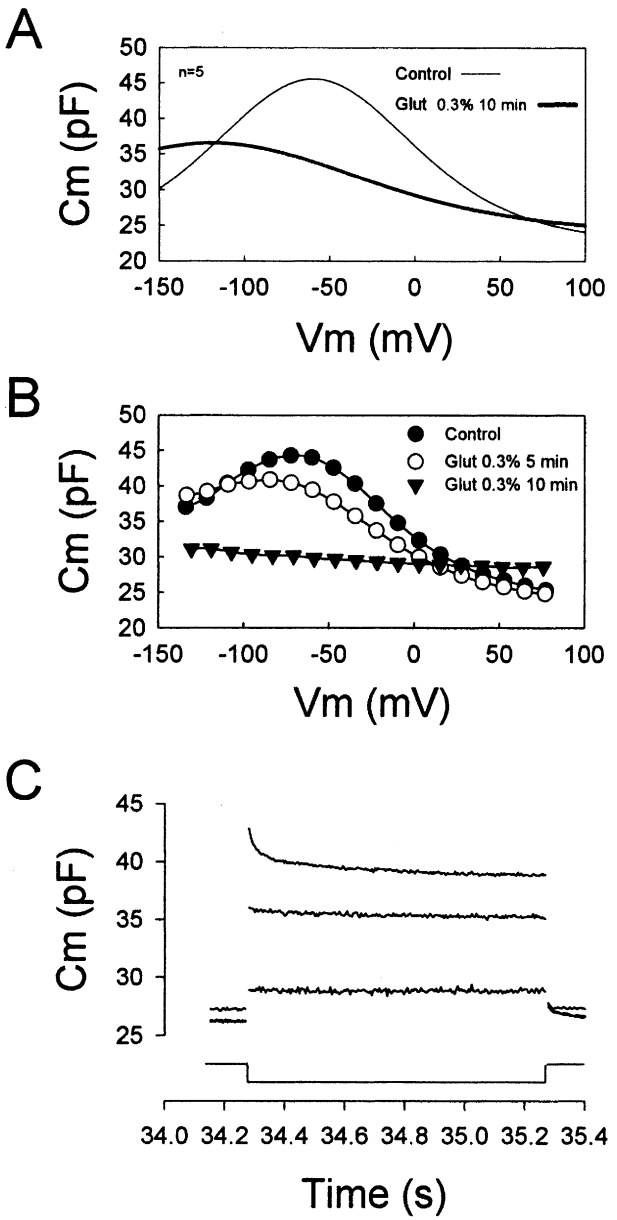

Fig. 1. Effect of low concentration (0.3%) of glutaraldehyde on OHC nonlinear capacitance. (*A*) *C-V* functions were obtained in five OHCs before and after 4.5 ± 1.1 min exposure to 0.3% glutaraldehyde. Fits with Eq. 1 were made and the estimated parameters were averaged. The resultant average fits (before and after treatment) are plotted. Control: Q_{max}, 3.60 ± 0.13 pC; V_{pkcm}, −59.6 ± 6.8 mV; z, 0.67 ± 0.06; C_{lin}, 22.6 ± 0.68; glutaraldehyde: Q_{max}, 3.1 ± 0.56 pC; V_{pkcm} −119 ± 12.4 mV; z 0.43 ± 0.04; C_{lin} 23.7 ± 1.42. (*B*) Nonlinear capacitance of an individual OHC is shown over time (0, 5, 10 min) following glutaraldehyde treatment. Note progressive shift in voltage dependence and decrease in capacitance. (*C*) Voltage step from a holding potential of +50 mV to −100 mV induces an instantaneous jump in capacitance followed by an exponential decrease in capacitance (*top trace*, control). Following glutaraldehyde treatment, this decay is abolished (*middle trace*, 5 min; *bottom trace*, 10 min). Same cell as in (*B*).

in capacitance (Fig. 1*C*, *bottom traces*), even at post-treatment times where substantial charge movement or NLC was evidenced.

Other protein-reactive agents altered OHC NLC to varying extents. Average results are presented for N-ethylmaleimide (NEM; 2 mM; n = 4), *p*-chloromercuriphenylsulfonate (pCMPS; 2 mM; n = 7), dithiolthreitol (DTT; 2 mM; n = 7) and diamide (2–5 mM; n = 7) (Fig. 2; *see* fit statistics in legend). Though the average NEM results showed little effect on V_{pkcm}, actually, of the four cells tested, two showed shifts to the left (−12.1 ± 2.9 mV), while the other two shifted to the right (20.9 ± 3.5 mV). Similarly, DTT caused a positive shift in 3 of 7 cells (10.7 ± 4.2 mV), while the remaining cells showed a negative shift (−11.1 ± 2.8 mV). pCMPS, like glutaraldehyde, was effective in substantially reducing nonlinear capacitance, but on average shifted V_{pkcm} in the positive direction. In 5 of 7 cells, pCMPS caused V_{pkcm} to shift in the positive direction (25 ± 4.5 mV); the 2 remaining cells showed a negative shift (−10.1 ± 1.4 mV). However, Fig. 3 illustrates, in the same OHC, that while nonlinear capacitance was reduced by pCMPCS, the amplificatory shift in V_{pkcm} remained essentially intact. This result is the same for DTT effects on the amplificatory shift, where comparing the C_m traces relative to pre- and post-treatment steady-state V_{pkcm} indicates similar relaxation phenomena (*see* Fig. 6 as well).

Lipid-reactive Reagents

Chloroform moderately reduced NLC and shifted V_{pkcm} to the left in all cells tested (n = 6) (Fig. 4*A*). The effects on the amplificatory shift, however, were profound (Fig. 4*B*). Compared to controls, the magnitude and time course of the voltage step-induced change in C_m were markedly decreased. Gating currents were only slightly affected by chloroform treatment, other than showing the expected effects of a shift in V_{pkcrn} (V_h), namely, changes in the magnitude of charge movement at a given voltage (Fig. 4*C*). Additionally, a slight reduction (~20–30%) in gating time constants was found. The effects of hexadecylphosphocholine (HePC; 2–10 mM, n = 10), filipin (0.75 mg/ml, n = 3), phospholipase A2 (PLA2; 1 ng/ml, n = 4), and triton (0.01%, n = 2) are illustrated in Fig. 5. Triton concentrations above 0.01 caused rapid and severe cell damage with loss of recordings. Only HePC significantly affected NLC, shifting V_{pkcm} in all cells tested to the right and decreasing peak capacitance slightly. Slight effects of HePC on the amplificatory shift were found, but in this case, it appears that the effects arose, as it did from DTT and pCMPCS (*see* above), from the shift in steady-state V_{pkcm}, since realigning the traces relative to V_{pkcm} results in corresponding traces whose characteristics

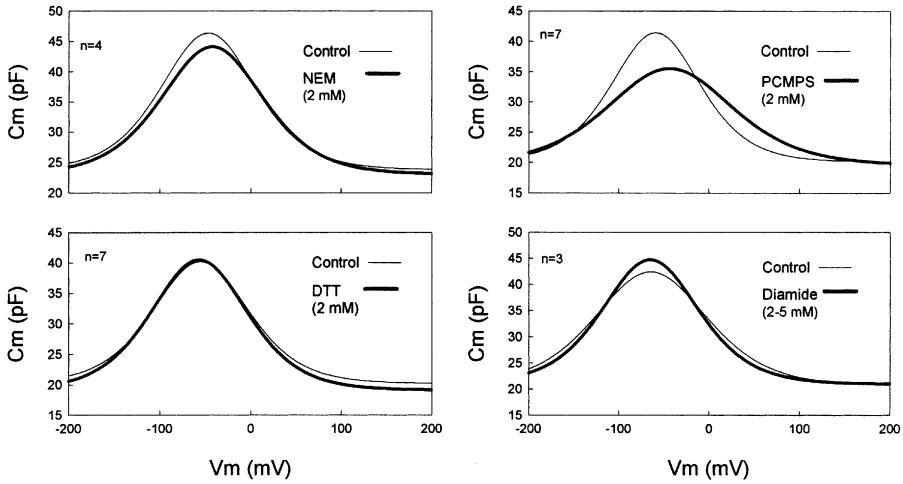

Fig. 2. Average OHC capacitance functions before and following treatments with NEM, pCMPS, DTT, and diamide. Data analyzed and presented as in Fig. 1*A*. pCMPS showed the greatest average effect. *NEM Control:* Q_{max}, 3.13 ± 0.59 pC; V_{pkcm}, −46.9 ± 11.5 mV; z, 0.74 ± 0.05; C_{lin}, 23.8 ± 1.6. *NEM* (3.12 ± 1.0 minutes): Q_{max}, 3.1 ± 0.3 pC; V_{pkcm}, −42.2 ± 6.1 mV; z, 0.69 ± 0.03; C_{lin}, 23.0 ± 0.8.DTT *Control:* Q_{max}, 2.82 ± 0.06 pC; V_{pkcm}, −55.2 ± 4.3 mV; z, 0.73 ± 0.02; C_{lin}, 20.2 ± 0.35; DTT (2.85 ± 0.3 minutes): Q_{max}, 3.03 ± 0.43 PC; V_{pkmc}, −56.9 ± 2.2 mV; z, 0.73 ± 0.02; C_{lin}, 19.13 ± 0.5. *pCMPS Control:* Q_{max}, 2.81 ± 0.18 pC; V_{pkcrn}, −59.0 ± 6.8 mV; z, 0.77 ± 0.03; C_{lin}, 20.1 ± 0.66; *pCMPS* (3.05 ±0.44 minutes): Q_{max}, 2.95 ± 0.25 pC; V_{pkcm}, −43.4 ± 8.5 mV; z, 0.55 ± 0.02; C_{lin}, 19.6 ±1.23. *Diamide Control:* Q_{max}, 3.64 ± 0.09 pC; V_{pkcm}, −64.4 ± 6.4 mV; z, 0.61 ± 0.03; C_{lin}, 20.6 ± 0.02; *Diamide* (5.43 ± 0.23 minutes): Q_{max}, 3.38 ± 1.58 pC; V_{pkcm}, −64.9 ± 4.6 mV; z, 0.72 ± 0.02; C_{lin}, 21.0 ± 0.6.

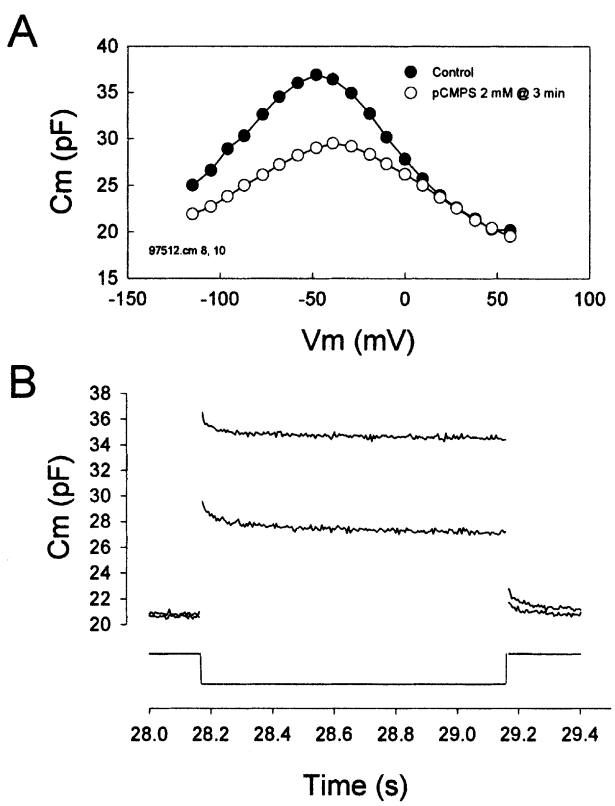

Fig. 3. Nonlinear capacitance of a single OHC before and after treatment with 2 mM pCMPS. (*A*) Following treatment, NLC is markedly reduced and right-shifted. (*B*) Voltage step from +50 to −100 mV induces an instantaneous jump in capacitance followed by an exponential decrease in capacitance. Little difference in the traces is observed except for the absolute offset magnitude.

are similar (Fig. 6; compare similarity in traces correspondingly labeled *1*, *2*, and *3*).

Temperature and Osmotic Swelling

Finally, we looked at the effects of temperature and membrane tension (osmotic cell swelling) on OHC capacitance. As we found previously, V_{pkcm} shifted to negative potentials and the magnitude of capacitance decreased with cooling (Santos-Sacchi & Huang, 1998). Interestingly, cooling also caused a decreased amplificatory shift (Fig. 7). On the other hand, membrane tension produced little effect on the amplificatory shift, despite showing the characteristic shift of V_{pkCm} to positive potentials (Kakehata & Santos-Sacchi, 1995) (Fig. 8).

Discussion

The OHC motor is an integral membrane protein that can be viewed as fluctuating between conformational states under the control of membrane voltage and chloride concentration (Santos-Sacchi & Dilger, 1988; Oliver et al., 2001; Rybalchenko & Santos-Sacchi, 2003). One model that can accurately account for mechanical and electrical activity of the OHC posits that the motor occupies two area states, contracted and expanded (Santos-Sacchi, 1993; Iwasa, 1994; Santos-Sacchi & Navarrete, 2002). Measures of NLC provide estimates of motor-state probability, and have been used to characterize the effects of a

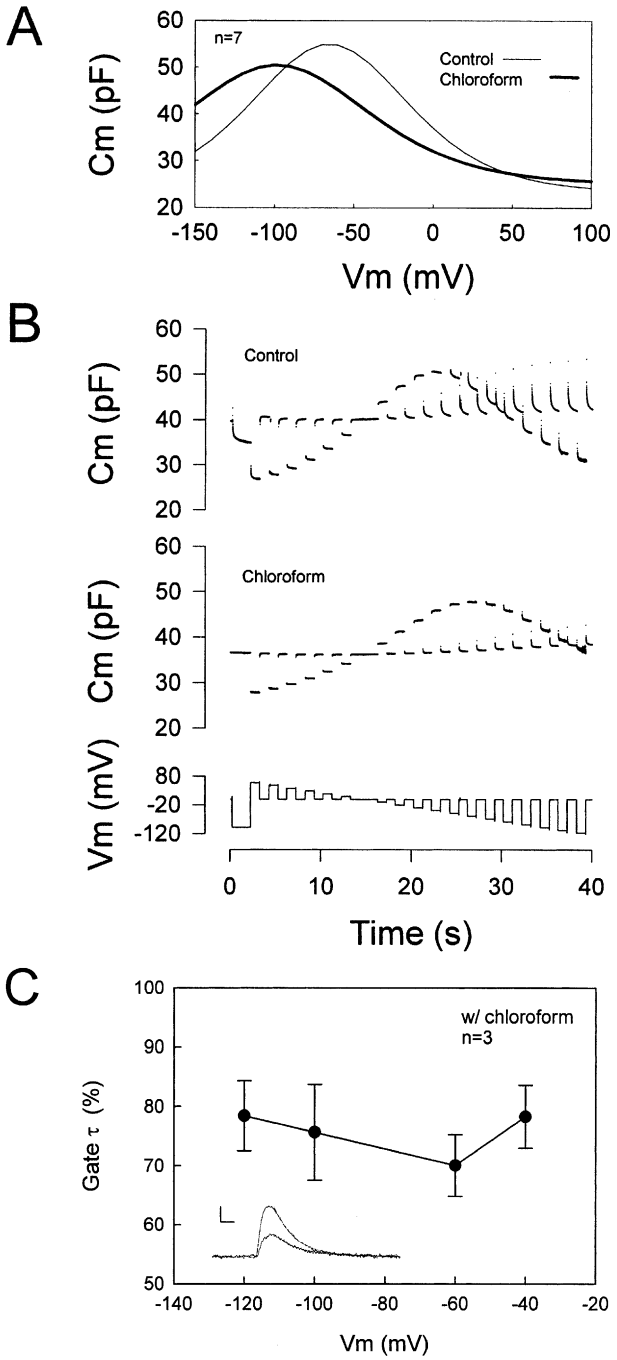

Fig. 4. Effect of chloroform on OHC nonlinear capacitance. (***A***) *C-V* functions were obtained in seven OHCs before and after exposure to a saturated chloroform solution. Fits with Eq. 1 were made and the estimated parameters were averaged. The resultant average fits (before and after treatment) are plotted. *Control:* Q_{max}, 4.21 ± 0.26 pC; V_{pkcm}, −65.4 ± 4.4 mV; z, 0.77 ± 0.09; C_{lin}, 23.3 ± 0.74; *Chloroform* (5.58 ± 0.39 minutes): Q_{max}, 4.0 ± 0.21 pC; V_{pkcm}, −98.5 ± 7.1 mV; z, 0.66 ± 0.02; C_{lin}, 25.0 ± 0.8. (***B***) Nonlinear capacitance of an individual OHC is shown during steps to a range of voltages (*lower panel*). Note large capacitance relaxations in control (*upper panel*) condition, and markedly attenuated response at all voltages in chloroform-treated condition (*middle panel*). (***C***) Average gating current time course (single exponential fits to the decaying current component) is slightly speeded up (~80% of control tau) after chloroform treatment. *Inset*: gating current traces from a single OHC obtained by P/-5 technique. Step from −120 to −80 mV. Larger trace is before treatment, but differences in magnitude largely result from the shift in V_h. Scale: 0.53 nA, 0.2 ms.

variety of biophysical forces on the motor including membrane tension and temperature (Iwasa, 1993; Gale & Ashmore, 1994, Santos-Sacchi & Huang, 1998, Santos-Sacchi & Navarrete, 2002). These forces are capable of shifting steady-state V_{pkcm}, the voltage where the population of motors is equally distributed between the two area states. Additionally, sensitivity to initial or prior voltage conditions manifests itself as a dynamic shift in the gating charge-voltage relation (*Q-V*; or correspondingly, the capacitance-voltage relation, *C-V*) along the voltage axis, which is time-and voltage-dependent (Santos-Sacchi et al., 1998). That is, membrane voltage change, while instantaneously altering the probability of the motors' occupying either the[2] contracted or expanded state, also influences future motor-state probability. Since a given polarity of voltage stimulation recruits additional motors into the same conformational state evoked by that eliciting voltage step, this dynamic shift in V_{pkcm} can be viewed as an amplificatory shift (Santos-Sacchi et al., 1998). This amplification event is an intrinsic characteristic of prestin, since this susceptibility to initial voltage is observed in non-auditory cells expressing prestin (Santos-Sacchi et al., 2001).

Our new data indicate that membrane-reactive agents can alter OHC electro-mechanical activity. Specifically, both lipid-and protein-altering drugs are capable of influencing the energy profile of the motor protein, prestin, as evidenced by shifts in the voltage at half-maximal charge movement (V_{pkcm}). Additionally, some of these agents have significant effects on the exponential amplificatory shift of V_{pkcm}.

Effects of Protein-reactive Reagents on the Motor

Low concentrations of glutaraldehyde blocked nonlinear charge movement presumably by interfering with conformational changes normally evoked by voltage. Yet, during the development of its effects, in the face of substantial nonlinear charge movement, the time-dependent change in capacitance during steady-state voltage step, namely the amplificatory shift, was abolished. This may indicate that the mechanism or molecular structure responsible for the amplificatory shift differs from the one underlying voltage sensing. Since we previously modeled the amplificatory shift as resulting from interactions

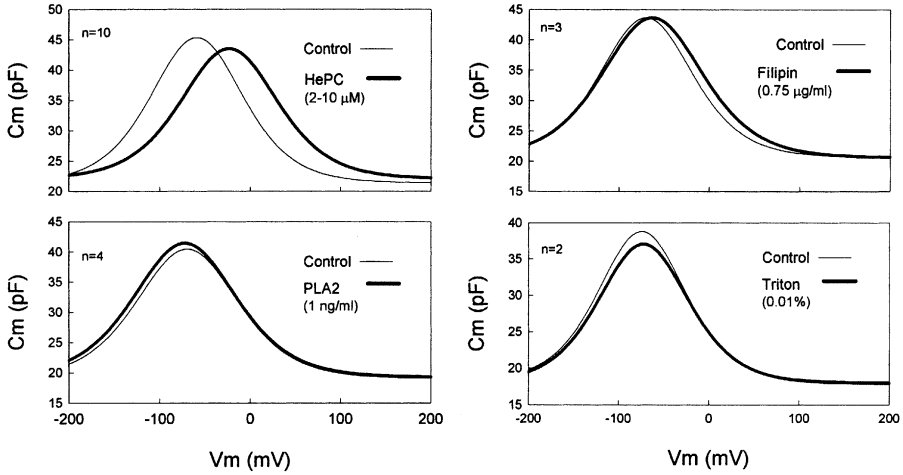

Fig. 5. Average OHC capacitance functions before and following treatments with HePC, Filipin, PLAZ and Triton. Data analyzed and presented as in Fig. 1*A*. HePC showed the greatest average effect. *HePC Control:* Q_{max}, 3.26 ± 0.24 pC; V_{pkcm}, −59.2 ± 4.4 mV; z, 0.76 ± 0.01; C_{lin}, 21.4 ± 1.6; HePC (4.89 ± 0.8 minutes): Q_{max}, 3.0 ± 0.1 pC; V_{pkcm}, −23.6 ± 9.3 mV; z, 0.72 ± 0.03; C_{lin}, 22.0 ± 0.9. *PLA2 Control:* Q_{max}, 3.19 ± 0.22 pC; V_{pkcm}, −69.8 ±8.6 mV; z, 0.69 ± 0.04; C_{lin}, 19.0 ± 0.7; *PLA2* (10.5 ± 3.2 minutes): Q_{max}, 3.36 ± 0.22 pC V_{pkcm}, 72.2 ± 7.7 mV; z, 0.68 ± 0.02; C_{lin}, 19.2 ± 0.7. *Filipin Control:* Q_{max}, 3.18 ±0.47 pC; V_{pkcm}, −69.9 ± 3.4 mV; z, 0.75 ± 0.03; C_{lin}, 20.6 ± 1.7; *Filipin* (3.0 ± 0.3 minutes): Q_{max}, 3.45 ± 0.3 pC; V_{pkcm}. −63.0 ± 12.7 mV; z, 0.69 ± 0.06; C_{lin}, 20.6 ± 2.2. *Triton Control:* Q_{max}, 2.69 ± 0.33 pC; V_{pkcm}, −74.2 ± 14.6 mV; z, 0.79 ± 0.13; C_{lin}, 18.1 ± 1.6; *Triton* (5.0 ± 1.0 minutes): Q_{max}, 2.56 ± 0.63 pC; V_{pkcm}, −72.3 ± 22 mV; z, 0.77 ±0.08; C_{lin}, 17.9 ± 0.6.

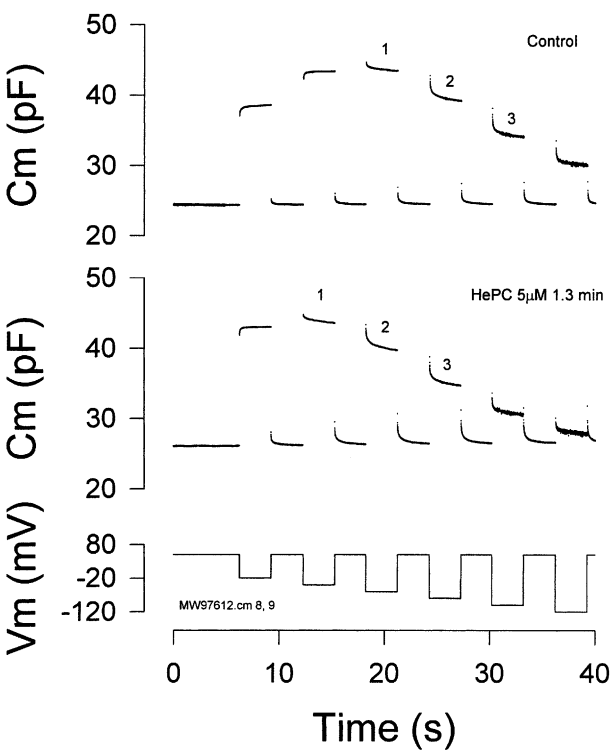

Fig. 6. Nonlinear capacitance of a single OHC during steps to a range of voltages before (*upper panel*) and after (*lower panel*) treatment with HePC. Following treatment, NLC is reduced and shifted in the depolarizing direction. Note large capacitance relaxations in control (*upper panel*) condition, which at any particular step voltage is modified by HePC. However, the similarity of relaxations at voltage step magnitudes relative to V_{pkcm}, i.e., comparing, for example, corresponding traces *1*, *2* and *3* in each panel, indicates that changes in relaxations may have resulted from the shift in V_{pkcm}.

among motors via motor-induced tension (Santos-Sacchi et al., 1998), it may be that glutaraldehyde can interfere with motor-generated tension within the bilayer.

Kalinec and Kachar (1993) studied the effects of sulfhydryl reagents on voltage-dependent OHC mechanical activity. Our complementary data on the lateral membrane's voltage-sensor mechanism confirm and extend their findings. Thus, they showed that pCMPS significantly reduced the percentage of mechanically active OHCs after 60 minutes incubation, but neither NEM nor diamide were significantly effective (Kalinec & Kachar, 1993). Similarly, we found that the sulfhydryl reagent pCMPS had the most potent effect on NLC, decreasing peak capacitance and shifting V_{pkcm} to depolarized levels. Whereas, in our hands, the effects of pCMPS were fairly rapid in onset, clearly occurring within 2–3 minutes of direct cell perfusion, Kalinec and Kachar (1993) found that significant effects on motor activity required greater than 20 minutes incubation. It is likely that their population measures, based on percent of motile cells, were not as sensitive as our single-cell gating-charge measures in determining the time course of effects. Consequently, our data may indicate that affected cysteine residues may reside at exposed sites within prestin. Based on current topology models (Zheng et al., 2001; Oliver et al., 2001; Adler et al., 2003; Navaratnam et al., 2004), three cysteine residues are predicted to lie external to the bilayer. In most models, only residue C415 resides extracellularly (Oliver et al., 2001; Zheng et al., 2001; Adler et al., 2003). However, the 10 transmembrane domain topology model of Navaratnam et al. (2004), predicts

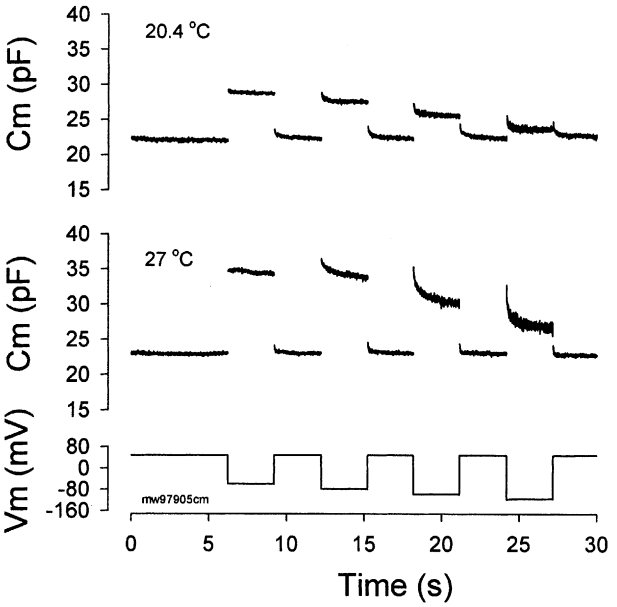

Fig. 7. Nonlinear capacitance of a single OHC during steps to a range of voltages at two different temperatures (*upper panel*: 20.4°C and *lower panel*: 27°C). Upon cooling, NLC is reduced but, in this case, there is little change in V_{pkcm}. However, C_m relaxations are markedly attenuated.

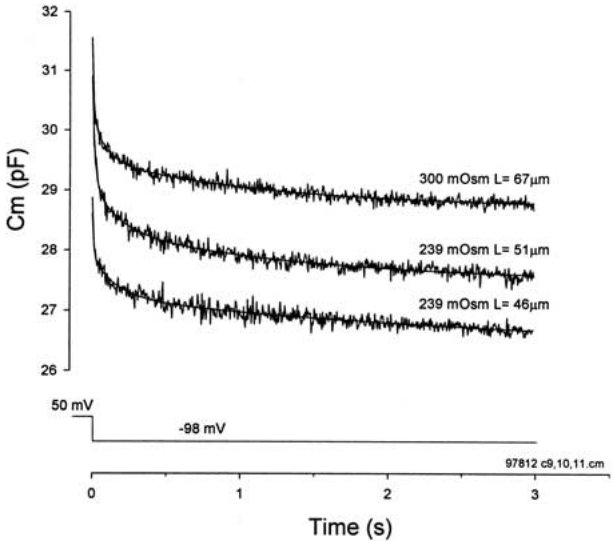

Fig. 8. Capacitance relaxations in a single OHC in response to a step voltage at increasing turgor levels. A switch from 300 mOsm to 239 mOsm caused a slow cell swelling, evidenced as cell shortening (length changed from 67 to 46 μm over a 5 min interval). Despite the increasing turgor pressure and marked shift of V_{pkcm} to hyperpolarizing levels (evidenced here as a reduction in baseline C_m magnitude) the C_m relaxation remains relatively unaltered.

that C124, and not C415, resides extracellularly. Thus it may be that pCMPS works mainly on only one extracellularly exposed cysteine residue in prestin. Nevertheless, supplementing any effects on these putative extracellularly exposed residues, it may be that pCMPS quickly enters the OHC, possibly via large aqueous channels in the lateral membrane (Morimoto et al., 2002; Rybalchenko & Santos-Sacchi, 2003), thereby working on intracellular cysteine residues. In all models, the two other cysteine residues are found within the N and C termini, which reside within the intracellular aqueous phase (C52, C679), and though their co-location may indicate that these residues could interact, the absence of a significant effect of the membrane-permeable monothiol stabilizing agent, DTT, on NLC indicates that such potential interactions are not required for normal function.

Diamide has a dramatic effect on OHC mechanical characteristics (Adachi & Iwasa, 1997). We found little effect on NLC with our short perfusions. However, since the effects of diamide on the OHC cytoskeleton (spectrin, in particular) may require prolonged incubations, we tested an additional 5 OHCs at incubation times of 1–1.5 h at 5 mM; neither overall charge movement nor amplificatory shift was significantly affected, indicating that the diamide-sensitive subsurface cytoskeleton has little impact on intrinsic motor activity, though it can have effects on whole-cell mechanical performance (Adachi & Iwasa, 1997). Similarly, it had been previously reported that digestion of the OHC cytoskeleton by intracellular enzymes did not adversely affect NLC (Huang & Santos-Sacchi, 1994; Santos-Sacchi et al., 1998) or mechanical activity (Kalinec et al., 1992).

Effects of Lipid-reactive Reagents on the Motor

Prestin is a protein predicted by topology mapping programs to reside substantially within the lipid bilayer (Zheng et al., 2001). Consequently, it is not surprising that we find significant effects of drugs that are known to modify the lipid bilayer. Notably, chloroform has marked effects on OHC charge movement, reducing peak capacitance and shifting the motor's operating range in the negative direction; its major effect, however, was on the amplificatory shift, reducing its magnitude while increasing its speed. This was observed across a wide voltage range, and cannot be accounted for by a simple steady-state shift in V_{pkcm}. OHC gating-current speed was little affected by chloroform, and this substantially derives from the limiting effect of the whole-cell-clamp time constant (Santos-Sacchi & Huang 1998).

The effects of chloroform on other integral membrane proteins have been studied. Chloroform reduces the magnitude of Na channel gating currents but does not affect their kinetics or their operating voltage (Fernandez, Bezanilla & Taylor, 1982). On the other hand, the kinetics of Shaker K channels were speeded up, though the operating voltage remained stable (Correa, 1998). In both cases ionic

currents were blocked. Interestingly, chloroform has been found to enhance K currents through two-pore-domain K channels (Patel et al., 1999). The effects on these ion channels and other integral membrane proteins have been suggested to arise from direct actions on the proteins themselves (Franks & Lieb, 1994). However, despite this possibility it is clear that chloroform does have substantial effects on the lipid bilayer since, for example, Fernandez et al. (1982) also showed by measuring displacement currents that the hydrophobic anion, dipicrylamine, more rapidly traverses the bilayer in the presence of chloroform. Thus, although it cannot be ruled out that the OHC motor is directly affected by chloroform, the lipid-altering capabilities of this agent, especially at the saturating concentrations used in this and the previously noted studies (Correa, 1998; Fernandez et al., 1982), indicate a likely lipid-based mechanism of action.

The only other lipid-reactive agent tested that significantly affected NLC was HePC, though the absence of major effects from some of the other treatments may have been due in part to the short exposure times that we employed. HePC is a phospholipid analog that can insert into lipid monolayers and interact with cholesterol (Rakotomanga et al., 2004). Although the operating range of the OHC motor is susceptible to HePC, the amplificatory shift was minimally affected, showing behavior that apparently resulted indirectly from the steady-state shift in V_{pkcm}. It should be noted, however, that regardless of the underlying reason for alterations in the amplificatory shift, such a change at a fixed resting potential can be physiologically important. Thus, it may be possible that lipids can modulate the time course of OHC mechanical activity, just as they are able to modulate the time course of ionic currents (Oliver et al., 2004). It should also be stressed that a simple steady-state shift in V_{pkcm} does not necessarily result in changes to the amplificatory shift. For example, membrane tension produces marked changes in steady-state V_{pkcm} but has little effect on the amplificatory shift at a fixed voltage.

Temperature Effects on the Motor

We have previously shown that OHC NLC is highly temperature sensitive, shifting steady-state V_{pkcm} to the left and reducing peak capacitance as temperature is decreased (Santos-Sacchi & Huang, 1998; Meltzer & Santos-Sacchi, 2001). However, we were unable to evaluate any changes in the speed of gating currents because the clamp time constant, even at 50 μs, under whole-cell mode was limiting. Effects of temperature on the amplificatory shift are clearly observed and a decrease in temperature markedly reduces the shift. Since temperature is known to affect both membrane proteins and lipids, it is possible that effects on motor activity may be dual.

Mechanisms of action on V_{pkcm}

The amplificatory shift has been modeled to result from interactions among motors via induced membrane tension (Santos-Sacchi et al., 1998). Whether the shift derives from motor interactions directly or via intervening lipid is an important issue, which can impact on OHC function. Thus, we have recently shown that functional motor microdomains may exist within the OHC, where local biophysical forces foster independent, local motor activity (Santos-Sacchi et al., 2001). The efficiency of coupling such forces, e.g., membrane tension, among motors could depend heavily upon the physical types of interactions responsible for motor coupling. For example, interactions via bilayer lipids may limit the distance over which motors could interact, whereas direct protein interactions could provide longer-range interactions. While our data may indicate that both lipid and protein moieties can influence the amplificatory shift, the high density of motor protein expression within the lateral membrane, estimated to be near $8000/\mu m^2$ (Huang & Santos-Sacchi 1993; Gale and Ashmore 1997), may significantly promote direct motor-motor interactions. In this regard, it would be interesting to test the effect of motor expression density on the amplificatory shift. Expression density is known to influence integral membrane protein function; for example, the fraction of non-inactivating CLC-0 channels is directly related to expression level (Pusch, Ludewig & Jentsch, 1997).

Regarding the steady-state shifts in V_{pkcm}, these modulations can not be due to changes in membrane viscosity, since forward and backward transition rates between protein conformations should be affected equally by changes in viscosity (Lee, 1991). Thus, based on a two-state model, temperature, chloroform and other lipid-modifying agents known to shift V_{pkcm} must instead alter the relative energy level of each motor state. Perhaps these manipulations act via perturbation of membrane tension or membrane bending, resulting in changes in conformational stability. Each of these phenomena has been implicated in motor activity (Iwasa, 1993; Gale & Ashmore 1994; Kakehata & Santos-Sacchi, 1995; Raphael, Popel & Brownell, 2000; Lue, Zhao & Brownell, 2001; Morimoto et al., 2002; Spector & Jean 2004). However, while viscosity is not likely to be involved in steady-state shifts, the rate of amplificatory shift induced by voltage steps might be expected to be affected. We suggest that chloroform may be working to reduce membrane viscosity, thereby speeding up the amplificatory shift.

Finally, we note that while prestin is considered to be essential for motor activity (Zheng et al., 2000;

Liberman et al., 2002), and the manipulations that we have made here may, in fact, alter prestin or its interactions with the lipid bilayer, it is not certain whether homo- or heteromeric configurations comprise the intact OHC motor (Santos-Sacchi, 2003). Thus, our manipulations may actually be working on players besides prestin. Indeed, we must remain cognizant that unexpected effects of the tested agents on mechanisms other than those we have highlighted may come into play, for example, altered anion binding. We are currently working on these issues (Navaratnam et al., 2004).

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