Plasticity in Membrane Cholesterol Contributes toward Electrical Maturation of Hearing

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Advances in refining the “fluid mosaic” model of the plasma membrane have revealed that it is wrought with an ordered lipid composition that undergoes remarkable plasticity during cell development. Despite the evidence that specific signaling proteins and ion channels gravitate toward these lipid microdomains, identification of their functional impact remains a formidable challenge. We report that in contrast to matured auditory hair cells, depletion of membrane cholesterol in developing hair cells produced marked potentiation of voltage-gated K⁺ currents (I_{Kv}). The enhanced magnitude of I_{Kv} in developing hair cells was in keeping with the reduced cholesterol-rich microdomains in matured hair cells. Remarkably, potentiation of the cholesterol-sensitive current was sufficient to abolish spontaneous activity, a functional blueprint of developing and regenerating hair cells. Collectively, these findings provide evidence that developmental plasticity of lipid microdomains and the ensuing changes in K⁺ currents are important determinants of one of the hallmarks in the maturation of hearing.

The matured auditory system is equipped with salient features that are essential for establishing the temporal fidelity required for speech and pitch perception (1). To achieve the post-hearing phenotype, pre-hearing hair cells undergo a precise and conserved transition from spontaneous action potentials (SAPs)² to graded generator potentials (2, 3). The importance of SAPs in the developing auditory system for synaptic refinement is reflected in the recapitulation of the preserved process in regenerating hair cells (4). Moreover, the electrical transition to graded receptor potential, which occurs at embryonic day (E) 18 in the chick and postnatal day (P) 12 in the mouse, coincides perfectly well with increased hearing sensitivity (3, 5–8). Previous studies have shown that several contributory conductances emerge and disappear in developing hair cells to culminate in the cessation of SAP. The functional expression of low voltage-activated K⁺ currents (I_{K,n}) derived from K₇.4 channels and Ca²⁺-activated K⁺ currents at later stages of development clamps the membrane potential close to the reversal potential of K⁺ (2, 3, 9, 10). Additionally, a reduction in the Ca²⁺ current density and the late-stage disappearance of low voltage-activated Ca²⁺ current (4) both contribute toward the transition from SAPs to graded receptor potentials, yet the underlying mechanisms for the late-stage functional appearance of K⁺ currents are unknown, despite evidence to demonstrate that K⁺ channel genes and products are expressed several days prior to hair cell maturation (11, 12).

A revised version of the structure of the plasma membrane consists of ordered microdomains rich in cholesterol, sphingolipids, and saturated phospholipids (13, 14), which may be organized by scaffolding proteins (15, 16). These cholesterol/ sphingolipid-rich domains are thought to confer spatial segregation of signaling pathways in cells (13). Indeed, several ion channels gravitate toward these cholesterol-rich “icebergs” in the sea of lipids, which regulate channel protein functions or their cell-surface expression (18, 19). Moreover, the composition of the lipid microdomains undergoes drastic and consistent plasticity during neurodevelopment, serving as stage-specific markers for certain lineages of cells (20–22). The plasma membrane of hair cells has been shown to contain lipid microdomains (23). Furthermore, hair cell membrane-bound motor proteins such as prestin and Myo1c have biochemical properties that are consistent with their association with lipid rafts (24–28), raising the possibility that lipid microdomains and their potential developmental plasticity play important roles in hair cell functions.

We report for the first time that depletion of cholesterol produces a differential modulation of voltage-gated K⁺ currents (I_{K,n}) in developing hair cells compared with matured cells. Significantly, the data are in keeping with the developmental plasticity of cholesterol in plasma membranes of developing and matured hair cells. The ensuing impact of cholesterol depletion is a profound attenuation of SAPs in developing hair cells. We propose that alteration of the lipid composition of membrane lipids is fundamental to the transition from SAPs to graded receptor potentials, an important feature in the maturation of hearing.

**EXPERIMENTAL PROCEDURES**

Isolation of the Chicken Basilar Papilla—This investigation was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of California Davis. Basilar papillae were isolated as described previously...
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(4). All experiments were performed within 5–45 min of isolation. This study included chickens at different stages of embryonic development, ranging from E6 and E21, as well as post-hatched chickens. Fertilized eggs were incubated at 37 °C in a Marsh automatic incubator (Lyon Electric). Before experiments, chicken embryos were killed and staged according to the number of somites present. Basilar papillae were isolated as described previously (4). The preparations were dissected in oxygenated chicken saline containing 155 mM NaCl, 6 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 5 mM HEPES, and 3 mM glucose (pH 7.4). The tegument vasculosum and the tectorial membrane were removed without any prior enzymatic treatment using a fine minutia needle. Chicken basilar papillae were stored in a 37 °C incubator in minimal essential medium (Invitrogen) before recordings from hair cell in situ. All experiments were performed at room temperature (21–23 °C) within 5–45 min of isolation. All of the reagents were obtained from Sigma unless specified otherwise.

**Electrophysiology**—K⁺ currents were recorded in a whole-cell voltage-clamp configuration using 3–5-megohm pipettes. Currents were amplified with an Axopatch 200B amplifier and filtered at 2 kHz through a low-pass Bessel filter, and data were digitized at 5–20 kHz using an analog-to-digital converter. This study included cells (n = 537) of healthy appearance whose leak current was not greater than 30 pA at a −80-mV holding potential throughout the recording period (~40 min). The sampling frequency was determined by the protocols used. No on-line leak current subtraction was made, and as such, only recordings with a holding current less than 20 pA were accepted for analyses. The liquid junction potentials were measured and corrected. The capacitative transients were used to estimate the capacitance of the cell as an indirect measure of the cell size. Capacitative decay was fitted with a single exponential curve to determine the membrane time constant. Series resistance was estimated from the membrane time constant, given its capacitance. The series resistances were within the 5–20-megohm range. After 60–90% compensation, the mean residual uncompensated resistance was 6.3 ± 0.5 megohms (n = 60). The seal resistance was typically 5–20 gigohms. Action potentials were amplified (100×), filtered (band pass 2–10 kHz), and digitized at 5–500 kHz as described (4). The stock solutions of channel blockers used were made in either double-distilled H₂O or dimethyl sulfoxide and stored at −20 °C. The final concentration of dimethyl sulfoxide in the recording bath solution was −0.001%.

**Recording Solutions and Drug Application**—The extracellular solution contained 125 mM NaCl, 6 mM KCl, 0–2 mM CaCl₂, 1.5 mM MgCl₂, 10 mM D-glucose, and 10 mM HEPES (pH 7.3, 310 mosm). The intracellular solution contained 120 mM KCl, 5 mM Na₂ATP, 2 mM MgCl₂, 10 mM HEPES, 2–5 mM BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid), and 10 mM D-glucose (pH 7.3). TEA, methyl-β-cyclodextrin (MβCD), and cholesterol were obtained from Sigma. The stock solutions of MβCD were made in dimethyl sulfoxide and stored at −20 °C. A cholesterol-MβCD (8:2) complex was prepared as described (29). Briefly, 100 mg of cholesterol was dissolved in 2 ml of 1:1 chloroform/methanol solution, and 12.8 µl of this solution was added to 10 ml of extracellular solution and left overnight at 37 °C. The solution was filtered to remove any remaining cholesterol crystals.

**Data Analysis**—The number of cells (n) is given with each data set. Data were analyzed using pClamp8 (Axon Instruments), Origin7.0 (MicroCal Software, Northampton, MA), and Excel 2000 (Microsoft, Redmond, WA). Voltage dependence of activation was examined from currents elicited by step depolarizations to potentials between −70 and 50 mV at different developmental stages (E10–P2), and then normalized curves were fitted with the Boltzmann distribution. Pooled data are presented as the mean ± S.D. Significant differences between groups were tested using Student’s t test, with p < 0.05 or 0.01 indicating a statistically significant difference. Data were analyzed using pClamp8 and Origin7.0. The n values reported reflect the number of cells.

**Histochemical Analysis**—To visualize cholesterol in the plasma membrane of hair cells, we used filipin staining (30). Filipin binds to cholesterol with high affinity and has natural fluorescence under UV excitation. Filipin (125 µg/ml) staining was performed in a light-protected room for 2 h at room temperature. After incubation with primary reagents, the preparation was washed with 0.5 M Tris containing no serum and counterstained with NeuroTrace™ 500/525 green fluorescent Nissl stain (N-21480, Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The preparation was visualized using a confocal microscope (Zeiss LSM 510).

**RESULTS**

**Methyl-β-cyclodextrin Increases I_{Kv} Only in Immature Auditory Hair Cells**—To examine the roles of membrane lipids in the functional development of ionic conductances in hair cells, we determined the sensitivity of voltage-gated K⁺ currents (I_{Kv}) to membrane cholesterol by application of the cholesterol-depleting compound MβCD. MβCD removes cholesterol from the plasma membrane and disrupts the function of lipid rafts in eukaryotic cell membranes (29). Whole-cell currents were elicited with ~250-ms depolarizing voltage steps in 10-mV increments from a holding potential of −80 mV. To suppress Ca²⁺-activated I_{Kv}, we omitted Ca²⁺ and included 100 nM iberiotoxin in the bath solution. Fig. 1A shows traces of I_{Kv} elicited from an E12 midsection basilar papilla hair cell before and after application of MβCD. MβCD significantly enhanced I_{Kv} as shown in Fig. 1B. The current-voltage relationship obtained from data from midsection basilar papilla hair cells (n = 16). The current modulated by MβCD or the cholesterol depletion-sensitive current is denoted as the difference current. The voltage dependence of current activation was not altered significantly, as shown in Fig. 1E. The Boltzmann fits are plotted with solid lines. Half-activation voltages were −31.3 ± 3.6 mV for the control and −35.1 ± 5.8 mV after application of MβCD (p = 0.3, n = 9). The maximum slope factors for the activation curves were 18.3 ± 1.8 mV and 16.8 ± 3.9 mV for control currents and after application of MβCD (p = 0.1, n = 9), respectively. In stark contrast to immature hair cells, the sensitivity of I_{Kv} to MβCD was lost in matured hair cells (Fig. 1C). Application of MβCD in P2 midsection hair cells left I_{Kv} unaltered, as illustrated in Fig. 1 (C and D).
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**FIGURE 1.** *MβCD* potentiates $I_{Kv}$ only in developing chicken hair cells. Currents were elicited by 250-ms depolarizing voltage steps in 10-mV increments from a holding potential of $-80$ mV. A, example of current traces recorded at E12 from the midsection of the basilar papilla. $I_{Kv}$ current traces recorded before and after the application of 1 mM *MβCD*. The difference current reflects the *MβCD*-sensitive component. B, current traces recorded at P2 from the midsection of the basilar papilla before and after the application of 1 mM *MβCD*. The difference current seen in A was abolished in P2 hair cells. C, the mean ± S.D. steady-state $I-V$ plots are also shown ($n = 16$). Note the increase in current amplitude with application of *MβCD*. D, in contrast, the $I-V$ plots for the current were unchanged after application of *MβCD* ($n = 12$). E, the Boltzmann fits were derived from the tail currents and plotted with solid lines from data obtained from hair cells at the midsection of E12 basilar papilla. Half-activation voltages were $-31.3 ± 3.6$ mV and $-31.1 ± 5.8$ mV ($p = 0.3, n = 9$) for control currents and after application of *MβCD*, respectively. The maximum slope factors for the activation curves were $18.3 ± 1.8$ mV and $16.8 ± 3.9$ mV ($p = 0.1, n = 9$) for control currents and after application of *MβCD*, respectively. F, summary data of the mean current density measured at 0-mV step potential from data collected from hair cells at the midsection of the basilar papilla (pA/picofarads (pF)) at E12, E16, E18, and P2. The sensitivity to *MβCD* decreased as hair cells became more mature. E12, $n = 16$; E16, $n = 14$; E18, $n = 13$; and P2, $n = 12$. *p < 0.05.

Density declined by $\sim 4$-fold from E12 to E18. *MβCD*-sensitive current density was $18.6 ± 2.1$ pA/picofarads at E12 and $4.6 ± 1.3$ pA/picofarads at E18. After E18, the *MβCD*-sensitive current disappeared. Of note, the density of $I_{Kv}$ increased by $\sim 3$-fold from E12 to P2.

**Methyl-β-cyclodextrin Exerts Its Effects via Membrane Cholesterol Depletion**—To determine the specificity of the *MβCD* on $I_{Kv}$, *MβCD* was saturated with cholesterol at a ratio of 8:2 (ratio shown to abolish *MβCD* lipid-buffering capacity (29, 31)). Application of a cholesterol/*MβCD* mixture did not potentiate $I_{Kv}$ in immature hair cells, as illustrated in Fig. 2. The magnitude of $I_{Kv}$ was unaltered after exposure to a cholesterol/*MβCD* 8:2 saturated complex (Fig. 2, A–D). Together, these experiments support the notion that *MβCD* exerts its effects on $I_{Kv}$ by cholesterol depletion of the plasma membrane of developing hair cells.

**Cholesterol Depletion Affects the TEA-sensitive $I_{Kv}$-delayed Rectifier**—*MβCD*-sensitive currents showed slow kinetics of activation and a lack of current inactivation (Fig. 1), raising the possibility that the delayed rectifier $I_{Kv}$ in developing hair cells is the targeted current. We tested the effect of TEA on *MβCD*-sensitive current (8). Fig. 3A (panels a–c) illustrates the effects of 5 mM TEA on the *MβCD*-sensitive $I_{Kv}$ recorded in E12 midsection basilar papilla hair cells. Note the increase in current amplitude after application of *MβCD* (Fig. 3, A and B) and subsequent current sensitivity to 5 mM TEA (Fig. 3A, panel c). The reverse experiment in which developing hair cells were pre-exposed to TEA showed no noticeable increase in current after exposure to *MβCD* (Fig. 3, C, panels a–c, and D). Thus, the data suggested that the TEA-sensitive $I_{Kv}$ is the *MβCD*-sensitive conductance. Because TEA-sensitive currents are present in adult hair cells (3, 8), it may be inferred that interaction of the channel producing the delay rectifier current with cholesterol is functionally relevant only in developing hair cells. Next, we cross-checked whether *MβCD* had any effect on inward Ca$^{2+}$ currents in developing versus matured hair cells. As shown in supplemental Fig. S1, Ca$^{2+}$ currents in hair cells were reduced after cholesterol depletion. However, the effects of *MβCD* were not statistically significant (supplemental Fig. S1).

**Changes in Membrane Cholesterol during Hair Cell Development**—To determine whether hair cell membrane cholesterol undergoes changes during development, we used filipin staining to evaluate cholesterol contents. Filipin labeling was markedly greater in E14 compared with P2 hair cells (Fig. 4). As demonstrated in Fig. 4, *MβCD* was effective in
depleting cholesterol in hair cells. If indeed these findings have any physiological relevance, we would predict that the magnitude of the TEA-sensitive $I_{Kv}$ would increase as part of the normal developmental plasticity of hair cell membrane cholesterol. The data shown in Fig. 1F are in keeping with the correlation between developmental changes in cholesterol and the magnitude of $I_{Kv}$, solidifying the physiological relevance of the present findings.

**Depletion of Membrane Cholesterol Has Profound Functional Consequences on Spontaneous Electrical Activity of Hair Cells**—To further investigate the functional ramifications of changes in membrane cholesterol in hair cell development, we examined the effects of MB/CD on the spontaneous electrical activity. Fig. 5 illustrates membrane SAP from immature midsection basilar papilla hair cells at E12 before (Fig. 5A) and after (Fig. 5B) exposure to MB/CD. Membrane depletion of cholesterol resulted in loss of spontaneous activity. The summary data on the effects of MB/CD on the resting membrane potential are shown in Fig. 5C. Alterations of MB/CD concentrations produced concentration-dependent changes in the spike frequency (Fig. 5D) and the resting membrane potential of hair cells, which is reminiscent of the effects of TEA on spike activity during development (3, 8). In addition, the spike width was reduced markedly (Fig. 5E).

**DISCUSSION**

In this study, we show that plasma membrane cholesterol is an important determinant of the magnitude of $I_{Kv}$ in hair cells. This effect of membrane cholesterol is an important functional feature specific to developing but not matured hair cells. If indeed these findings have any physiological relevance, we would predict that the magnitude of the TEA-sensitive $I_{Kv}$ would increase as part of the normal developmental plasticity of hair cell membrane cholesterol. The data shown in Fig. 1F are in keeping with the correlation between developmental changes in cholesterol and the magnitude of $I_{Kv}$, solidifying the physiological relevance of the present findings.

**FIGURE 2.** $I_{Kv}$ currents in developing hair cells are resistant to the cholesterol-MB/CD complex. Current traces were obtained from E12 hair cells from the midsection of the basilar papilla. Currents were elicited as described in Fig. 1. Shown are the current traces and amplitude for control conditions (A) and after exposure to the cholesterol-MB/CD 8:2 saturated complex (B). C, the difference current traces reflect the loss of MB/CD effects seen in Fig. 1. D, summary data of the I-V relationship obtained from 12 hair cells at E12 from the midsection of the basilar papilla. The data suggest that MB/CD exerts its effects on $I_{Kv}$ by depletion of membrane cholesterol.

**FIGURE 3.** MB/CD modulates TEA-sensitive $I_{Kv}$ in developing chicken hair cells. A, exemplar current traces recorded at E12 from the apical aspects of the basilar papilla. $I_{Kv}$ currents were recorded before (panel a) and after (panel b) the application of 1 mM MB/CD, followed by application of 5 mM TEA plus 1 mM MB/CD (panel c). The difference current (panel b-a) reflects the MB/CD-sensitive component, and panel b-c reflects the TEA-sensitive component (panels b and c). B, summary data of steady-state I-V plots are also shown (n = 11). Note the increase in the current amplitude with application of MB/CD and subsequent current sensitivity to 5 mM TEA. C, example of current traces recorded at E12 from the midsection of the basilar papilla. $I_{Kv}$ currents were recorded before (panel a) and after (panel b) the application of 5 mM TEA, followed by the application of 1 mM MB/CD plus 5 mM TEA (panel c). The difference current (panel a-b) reflects the TEA-sensitive component, panel c-b clearly demonstrates that the MB/CD-sensitive component was abolished. D, summary data showing the steady-state I-V plots (n = 9). Note that in the presence of TEA, there is a loss of MB/CD-sensitive current.
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The composition of membrane lipids greatly affects the biophysical and mechanical properties of the plasma membrane such as the fluidity and stiffness, which can modulate the gating of voltage-gated channels (35–38). Additionally, cholesterol may exert its effect by directly binding to the membrane proteins, affecting protein conformation and dynamics (39, 40). Single-channel studies have eliminated the likelihood that membrane lipids modulate single-channel unitary conductances, thereby potentiating the current (28, 41). Instead, it appears that cholesterol affects the number of active channels in the plasma membrane. In this study, the relatively short delay between MβCD application (∼2 min) and the effect on $I_{Kv}$ precludes the possibility for changes in channel synthesis or subunit assembly. However, there are several reports indicating that the membrane cholesterol modulates the equilibrium between active and silent forms of channels (28, 31, 41). Alternatively, it is conceivable that cholesterol modulates the surface distribution of the channels, which, as it turns out, has marked impact on channel functions (31, 42). Furthermore, in addition to direct interactions with membrane cholesterol, the observed potentiation of $I_{Kv}$ in developing hair cells could ensue from indirect association of $K_v$ channels with other binding proteins that are lipid-sensitive (33, 43, 44). Moreover, cholesterol depletion eliminates SAPs in developing hair cells, and in keeping with the roles of $K_v$ channels, the resting membrane potential and action potential durations were altered accordingly.

Lipid microdomains represent important niches for compartmentalization of cell functions. Indeed, several essential features of hair cells are derived and made possible by exclu-
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