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Motile Responses of Cochlear Outer Hair Cells Stimulated with an Alternating Electrical Field

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Abstract

The goal of the present study was to evaluate and characterize the motile responses of guinea pig OHCs, stimulated at frequencies varying from 50 Hz to 4 kHz, using high-definition, high-speed video recording and fully automatic image analysis software. Cells stimulated in continuous, burst and sweeping modes with an external alternating electrical field showed robust fast and slow motility, which were dependent on frequency, mode and intensity of stimulation. In response to continuous stimulation, electromotile amplitude ranged from 0.3 % to 3.2 % of total cell length, whereas cell length usually decreased in amounts varying from 0.1 % to 4.3 %. Electromotile amplitude in OHCs stimulated with square wave's sweeps was near constant up to 200 Hz, progressively decreased between 200 Hz and 2 kHz, and then remained constant up to 4 kHz. In continuous and burst modes electromotility followed cycle-by-cycle the electrical stimulus, but it required 1 to 2 s to fully develop and reach maximal amplitude. Instead, slow cell length changes started about 0.6 s after the beginning and continuously developed up to 3 s after the end of electrical stimulation. Incubation of OHCs with 10 mM salicylate affected electromotility but not slow motility, whereas incubation with 3 mM gadolinium affected both. Thus, combination of external electrical stimulation, high-speed video recording and advanced image analysis software provides information about OHC motile responses at acoustic frequencies with an unprecedented detail, opening new areas of research in the field of OHC mechanics.

Keywords

OHC motility; alternating electrical field; high frequency stimulation; cochlea; guinea pig

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1. Introduction

Outer hair cells (OHCs) transduce electrical signals into changes in length by activating a plasma membrane-based force generator mechanism associated with conformational changes in the integral membrane protein prestin (Ashmore, 2008; Dallos et al., 2002). Electrical stimulation is also able to induce changes in OHCs' length by activating a prestin-independent mechanism associated with cytoskeletal reorganization (Ashmore, 2008; Matsumoto et al., 2005a; Matsumoto et al., 2005b; Matsumoto et al.). The former cellular response is known as OHC electromotility, fast motility or prestin-dependent motility, whereas the latter is known as slow motility or prestin-independent motility.

Whole-cell voltage clamp has been the prevalent technique for investigating OHC electromotility ever since the pioneering studies of Ashmore in the mid 1980's (Ashmore, 1987). However, voltage clamp limited frequency response makes it ineffective at the kHz range, the key domain for acoustic stimuli (Santos-Sacchi, 1992). An alternative approach, termed the microchamber (Dallos et al., 1993; Evans et al., 1989; Evans et al., 1991), bypasses patch-clamp limitation by using suction pipettes. OHCs can be drawn to varying extents into a suction pipette with an internal diameter closely matching the cell diameter. As current is passed down the pipette it hyperpolarize (or depolarize) the membrane within the microchamber and depolarize (or hyperpolarize) the externally exposed membrane. OHC motility is evaluated using fast response photodiodes aligned on optical boundaries of the image of a region of the hair cell outside the pipette, usually the tip of the stereocilia or the apical surface of the cuticular plate. Using this technique, Dallos and colleagues showed that electromotile amplitude, after an initial decrease, remains almost constant from frequencies about 1 kHz up to at least 22 kHz (Dallos et al., 1995a; Dallos et al., 1995b). With the same basic technique but using a laser vibrometer to measure light reflected from an atomic force microscope cantilever placed on the cell axis, Frank et al demonstrated that OHCs were able to generate a near constant force up to 79 kHz at room temperature (Frank et al., 1999). Unfortunately, since simultaneous measurements of OHC's length, area or volume were not possible because of the particular experimental setups, the contribution of slow motility was not evaluated in these experiments. As electromotile amplitude is directly proportional to the total length of the OHC, true changes in electromotile amplitude can be reliably estimated only if variations in cell length associated with slow motility are taken in account. Consequently, although these experiments were singularly important for providing the first indisputable evidence that OHCs were indeed able to move at high frequencies following cycle-by-cycle the electrical stimulation, they did not provide a comprehensive description of the changes in shape associated with OHC motile responses.

The goal of the present study was to evaluate and characterize the motile responses, electromotility- as well as slow motility-induced changes in shape, of guinea pig OHCs electrically stimulated via an external alternating electrical field (EAEF) at frequencies varying from 50 Hz to 4 kHz. Although exposure to EAEFs was the initial approach used to investigate OHC motility (Brownell et al., 1985), it was soon replaced by the patch-clamp and microchamber methodologies and only a handful of additional studies with this technique were published in the last twenty years (Frolenkov et al., 1997; Gitter et al., 1995; Iwasa et al., 1989; Kachar et al., 1986; Ohnishi et al., 1992). Now, with the recent introduction of ultra-fast, high-definition video recording systems, together with image analysis software that facilitates the automatic one-by-one analysis of the hundred of thousands of frames in these recordings, a new spectrum of possibilities is open for the EAEF approach. The simplicity of this technique, and the opportunity to investigate OHC motility at frequencies well above the limit for patch-clamp studies and without some of the limitations and uncertainties associated with the microchamber method, may result in the return of this methodological approach to the center stage in OHC motility research.

In addition to providing the first, to our knowledge, comprehensive description of the mechanical responses elicited in OHCs by electrical stimulus in this range of frequencies, the results of the present study further extend our understanding of the cellular and molecular mechanisms responsible for OHC motility and open new areas of research in OHC mechanics.

2. Material and Methods

2.1. Isolation of OHCs

Cochleae were obtained from albino guinea pigs (*Cavia porcellus*, 200-300 g) of either sex euthanized with CO₂ following procedures approved by the House Ear Institute's Institutional Animal Care and Use Committee. The organ of Corti was removed from cochlear spiral in Leibowitz L-15 (Gibco-Invitrogen, Gaithersburg, MD), and incubated with 1mg/ml collagenase (type 4, Sigma, St. Louis, MO) at room temperature for 5 min. OHCs were mechanically dissociated by reflux through the needle of a 50 μ l Hamilton syringe. Isolated OHCs suspended in L-15 were transferred to a recording chamber (PCCS1, Bioscience Tools, San Diego, CA) placed on the rotating stage of an Axiovert 135TV inverted microscope (Zeiss, Thornwood, NY), and observed with Nomarski differential interference contrast optics with a 63x/1.2 C-Apochromat objective. Osmolarity was measured with a μ Osmette 5004 freezing-point osmometer (Precision Systems, Natick, MA), and adjusted to 305-310 mOsm by adding distilled water to L-15. Only OHCs ranging from 62.4 μ m to 79.6 μ m in length and complying with established health criteria (e.g., cylindrical shape, no swelling or other distortion of the cell membrane, and nucleus placed near the base of the cell (Zajic et al., 1987)) were used in this study within 2 h of isolation.

2.2. Electrical stimulation

Only OHCs without any attachment to the surface of the recording chamber were used in the reported experiments, since this attachment could alter the EAEF-induced movement (Frolenkov et al., 1997). Single OHCs were placed between two 0.25 mm-diameter Ag electrodes with tip distance of 0.8 mm (Fig. 1).

Cell's longitudinal axis was kept parallel to the applied EAEF by rotating the recording chamber. To measure motile amplitude one electrode was connected to ground and the other was stimulated with square wave pulses of 4, 8, 12 or 16 Volts peak-to-peak (Vpp) generated by a waveform generator with 50 Ω of output resistance (HP33120A, Hewlett-Packard, Loveland, Co). The custom-made control circuitry, developed by the Engineering Core of the House Ear Institute, is bus-powered through the USB port of a PC and consisted of a trigger generator, stimulation enable circuit and trigger selection circuit. The EAEF control software, also developed by the Engineering Core of the House Ear Institute, allows the user to specify stimulation in terms of shape, type, frequency, and amplitude. The stimulation duration and its shape can also be selected. In this study three different stimulation protocols were used: i) single frequency continuous, ii) single frequency bursts (100 ms on/100 ms off), and iii) 50 Hz-4 kHz square wave sweeps (Fig. 2). A Video Article with more details about this technique is being published elsewhere (Kitani et al., 2011).

Each OHC was stimulated only once and, except were specifically noted, stimulation was applied only for one period of 3 s. The electrical field was mapped using an additional electrode consisting of a silver wire covered with a 1 μ m-diameter pipette made from borosilicate capillary glass (TW150-4, World Precision Instruments, Sarasota, FL) using P-97 micropipette puller (Sutter Instruments, Novato, CA) (Fig. 3 A). EAEF strength was proportional to electrode stimulation, and ranged roughly from 4 to 20 V/cm at the center of the imaginary line connecting both electrodes (Fig. 3 B and C).

2.3. Image capture and data handling

Images of isolated OHCs were captured in AVI format at high frequencies (1,000 through 18,000 fps) and high resolution (1024×1024 pixels at 1,000 fps, 512×128 at 10,000 fps, and 384×96 at 18,000 fps. See Supplemental Information) using the ultra-high speed Photron Fastcam X 1024 PCI camera (Photron USA Inc.) and a LED-based illumination system (High Power LED System-36AD3500, Lightspeed Technologies, Campbell, CA) (Kitani et al., 2011). The LED illumination system was digitally synchronized with the high-speed camera, working like a stroboscopic flash going on and off with the aperture/closing of the camera shutter. In this way we minimized the changes in sample temperature associated with the illumination system. The high-speed camera, the LED-based illuminations system and the EAEF generator were all computer-controlled through an interface specifically designed to integrate different components of the system. In a typical experiment, once the parameters are chosen, the user hits a button to configure the generator with the selected parameters and a start button is enabled in the computer screen. This start button in the computer screen activates the output of the trigger pulse and the stimulus. The trigger pulse occurs 500 ms before the first stimulus pulse is delivered to the cells, so that the camera is already recording when the first pulse is delivered (Kitani et al., 2011). In every case the recording speed was selected to comply with the Nyquist criterion, that is, the number of frames per second was always more than double the highest frequency of electrical stimulation. Cell's images were analyzed off-line using ProAnalyst software (Xcitex Inc., Cambridge, MA) and further processed using Excel software (Microsoft, Redmond, WA). Measurements were always performed between points belonging to the same cell image. Thus, they were independent of any external frame of reference and consequently unaffected by potential rotation or lateral displacements of the cells induced by ionic currents in the surrounding fluid. All morphological parameters were normalized to initial values, e.g. OHC electromotile amplitude (difference in length between the contracted and elongated states) was expressed as a percentage of the total cell length at rest. Under our experimental conditions, changes in cell length as low as 26 nm were reliably detected (Fig. 4). This resolution was further improved with standard statistical methods (Frolenkov et al., 1997).

2.4. Pharmacological agents

Salicylate and Gd^{3+} (Sigma) were used as inhibitor of prestin function and non-selective cation channels blocker, respectively (Kakehata et al., 1996). Salicylate and Gd^{3+} were added at final concentrations of 10 mM and 3 mM, respectively, to an extracellular solution containing (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, with pH adjusted to 7.4 with Tris-OH and osmolarity to 305-310 mOsm with D-glucose. Isolated cells were incubated in this solution for 3 min before electrical stimulation.

2.5. Statistical Analysis

ANOVA techniques and JMP 9 statistical software (SAS Institute, Cary, NC) were used to analyze the data, and $p < 0.05$ was selected as the criterion for statistical significance. Curve fitting for values of electrical potential at different frequencies (Fig. 6) was automatically performed using SigmaPlot 11 (Systat Software, San Jose, CA).

3. Results

3.1. Relationship between EAEF strength and electrode placement with OHC motile response

We investigated the effects of EAEF strength on the motile responses of isolated guinea pig OHCs using a 50 Hz continuous single frequency protocol. We found that electromotile

amplitude and slow changes in OHC length increased when the magnitude of electrical stimulation was varied from 4 Vpp to 12 Vpp (Fig. 5 A). However, for stimulations below 4 Vpp the induced electromotile and slow motility responses were very small, and for magnitudes above 12 Vpp they did not increase and some cells showed signals of deterioration (e.g., disruption of the basal end membrane). Thus, in subsequent experiments we used 8 Vpp, which induced robust motile responses without any detectable sign of cell damage and no visible electrophoretic flow in the fluid nearby the floating OHCs.

Our study confirmed previous results (Gitter et al., 1995; Iwasa et al., 1989) indicating that the cuticular plate always moves toward the positive electrode during cell elongation and backward from the negative at cell contraction, with the basal pole always motioning in anti-phase (Fig. 5 B). In our experiments, in contrast to previous studies, OHCs were not attached to the culture dish, suggesting that this response was associated with an intrinsic characteristic of the OHCs and not with a reorganization of the cortical cytoskeleton triggered for the formation of focal adhesions or stress fibers. Since all our measurements were independent of any frame of reference external to the OHCs, our studies were not affected either by any potential displacement of the cells associated with electrophoretic flow of the surrounding fluid induced by the applied EAEF.

3.2. Electrode Polarization

We investigated the effect of electrode polarization on the electrical field generated by an electrical stimulation of 8 Vpp, at the full range of frequencies covered in our experiments, by mapping the electric potential using additional electrodes. Our results are summarized in Fig. 6.

The electrical field strength at any point in the line connecting the center of both electrodes was closer to 8 V/cm, specifically 7.75 V/cm, at 50 Hz, but it decreased with frequency (Fig. 6A). Highest values of electric potential were recorded at 50 Hz, and decreased with frequency at an average ratio of 0.04 ± 0.02 mV/Hz. A detailed inspection of the data associated with Fig. 6A indicated that this ratio was 0.13 ± 0.13 mV/Hz for frequencies below 100 Hz, decreased to 0.033 ± 0.008 mV/Hz for frequencies between 100 Hz and 500 Hz, increased again to 0.11 ± 0.03 mV/Hz for frequencies between 500 Hz and 1 kHz, and then decreased in a progressive manner up to a value of 0.020 ± 0.001 mV/Hz between 3 kHz and 4 kHz. We found a similar response when analyzing the EAEF strength (Fig. 6B). The total change in EAEF strength between 50 Hz and 4 kHz was 2.250 ± 0.001 V/cm, but the initial ratio of 5.0 ± 2.9 mV/cm per Hz decreased to 0.31 ± 0.18 mV/cm/Hz between 100 Hz and 500 Hz, increased again to 1.25 ± 0.14 mV/cm/Hz between 500 Hz to 1 kHz, and decreased to 0.62 ± 0.10 mV/cm/Hz between 1 kHz and 3 kHz and to near zero for frequencies between 3 and 4 kHz.

Next, we analyzed the effect of this change in EAEF strength on OHC motile responses by measuring electromotile amplitude and total cell length at different frequencies while maintaining constant the electric potentials and the electric field strength experienced by the cells. As shown in Fig. 6C, the corrected values of electromotile amplitude were not significantly different from the original ones, showing similar dependence on frequency. Differences were undetectable at frequencies below 1 kHz, whereas for frequencies between 1 kHz and 4 kHz the estimated increase in amplitude, statistically no significant, was always lower than 0.2 %.

Except electrode polarization, we detected no evidence of any other electrochemical phenomenon with spurious effects on cell motile responses.

3.3. Continuous single frequency stimulation

OHCs' responses to continuous stimulation were frequency-independent in every aspect except magnitude, which was roughly inversely proportional to the frequency. Electromotile amplitude gradually increased with time, reaching a plateau about 1 s after start of stimulation (Fig. 7).

We found this behavior typical for OHCs not stimulated for periods of 5 min or longer, whereas the time required to reach the plateau decreased for shorter periods and was zero for periods of no stimulation shorter than 1 s (results not shown). Electromotile amplitude at a frequency of 50 Hz was 2.4 ± 0.2 % (mean $\pm$ SE; $n=6$), with a peak value of 3.3 %. It decreased for higher frequencies, with values of 1.6 ± 0.2 % at 1 kHz ($n=6$; $p < 0.02$ vs. 50 Hz) and 0.9 ± 0.3 % at 4 kHz ($n=4$; $p < 0.0006$ vs. 50 Hz, NS vs. 1 kHz). Slow changes in cell length were observed to develop and continue for 0.5 to 3 s after the end of electrical stimulation, but simultaneous presence of electromotile responses precluded the precise estimation of the moment they started. Adopting as final cell length the value obtained 3 s after the end of electrical stimulation we found EAEF-induced decreases in cell length of 1.8 ± 0.5 % at 50 Hz, 0.8 ± 0.5 % at 1 kHz, and 0.4 ± 0.6 % at 4 kHz, with no significant statistical differences between them.

3.4. Linear sweep stimulation

OHCs stimulated with linear sweeps from 50 Hz to 4 kHz, and from 4 kHz to 50 Hz in control experiments (not shown), showed clearly the frequency-dependence of electromotile amplitude (Fig. 8).

Because of the 1 to 2 s period required for electromotile amplitude to fully develop (Fig. 7 B), every linear sweep experiment was preceded by an initial stimulation with a continuous EAEF of 50 Hz for a period of 1.5 s, and a subsequent gap of 600 ms required for the automatic reprogramming of the wave generator. As shown in Fig. 8, A and B, electromotile amplitude was near constant up to 200 Hz, progressively decreased between 200 Hz and 2 kHz, and then remained near constant until the end of the experiment at 4 kHz. This response was similar for electrical stimuli of 4 ($n=4$), 8 ($n=5$), 12 ($n=4$) and 16 ($n=3$) Vpp but, interestingly, while electromotile amplitude was significantly lower at 4 Vpp than at 8, 12 or 16 Vpp for all frequencies, at 8 Vpp it was lower than at 12 and 16 Vpp only between 50 and 200 Hz, whereas no significant differences in electromotile amplitude for electrical stimuli of 12 and 16 Vpp were found for any of the frequencies investigated. (Fig. 8 B). The absence of significant differences in electromotile amplitude at frequencies above 1 kHz with 8 Vpp, 12 Vpp and 16 Vpp electrical stimuli confirmed our previous results (section 3.2) about the lack of significant effects of electrode polarization on OHC electromotility. Actual values of EAEF strength (no corrected for electrode polarization) for 12 Vpp and 16 Vpp electrode stimulation were higher than 8 V/cm strength but induced no significant differences in electromotile amplitude or total cell length.

3.5. Stimulation with single frequency bursts

Isolated OHCs were stimulated with 100 ms bursts of 50 Hz ($n=8$), 1 kHz ($n=4$) and 4 kHz ($n=3$). For 50 Hz stimuli electromotile amplitude increased with time, reaching a plateau at values of up to 2.3 % of the total cell length after being stimulated for approximately 2 s (Fig. 9). For higher frequencies, however, cells required more time to reach the plateau, and for frequencies of 1 kHz and above it was not observed in the period covered by our experiments. Therefore, although we recorded top values of 1.3 % for 1 kHz and approximately 0.8 % for 4 kHz (Fig. 9 C), we were not able to establish the highest value for electromotile amplitude at these frequencies. After 0.6 s of stimulation electromotile

amplitude at 50 Hz was always significantly higher than at other frequencies, whereas differences between 1 kHz and 4 kHz were never statistically significant.

This experimental protocol, in contrast to continuous stimulation, allowed us to establish that slow motility started about 0.6 s after the beginning of stimulation (Fig. 9 B, arrow), reaching values of up to 1.8 ± 0.3 % of total cell length at 3 s for a frequency of 50 Hz. For higher frequencies cell shortening became progressively slower, showing biggest contractions of only 0.8 ± 0.4 % at 1 kHz and 0.39 ± 0.07 % at 4 kHz after 3 s of stimulation (Fig. 9 B). After 1.8 s of stimulation, contractions were significantly bigger in OHCs stimulated at 50 Hz than at 1 kHz and 4 kHz, whereas differences between 1 kHz and 4 kHz were never statistically significant.

As illustrated in Fig. 9 A, cell elongation associated with electromotility was always larger than cell contraction, a behavior already reported (Frolenkov et al., 1997). However, after slow motility started to develop, cell contraction amplitude increased proportionally more than cell elongation. For example, at 50 Hz, the ratio elongation/contraction decreased 20-fold, from ~ 40 at 0.2 s to 2.1 at 2.9 s (Fig. 9 A).

3.6. Changes in OHC area and volume during EAEF stimulation

The image system used in this study, in particular the image analysis software, enables a quite precise evaluation of stimulation-induced changes in cell length and two-dimensional (projected) area of isolated OHCs. Thus, it was possible to evaluate the applicability in studies with EAEF of the relationship linking relative changes in length, area and volume associated with OHC motility derived by Matsumoto and Kalinec (Matsumoto et al., 2005a). As predicted by the theory, we found that A^2 provided a very good description of the contribution of electromotile responses, whereas L/A^2 (equivalent to V^{-1}) made the same for slow OHC motility (Fig. 10).

Measurements of A , in particular, were more precise than in patch-clamped cells, where the tip of the patch pipette is an additional source of error because of its effect on the automatic definition of the cell boundaries (see Fig. 1 in (Matsumoto et al., 2005b)). Thus, combination of high-speed recording cameras with the EAEF technique might become a simpler and powerful alternative for investigating morphological changes in OHCs associated with their motile responses.

3.7. Effects of salicylate and Gd^{3+}

Salicylate and Gd^{3+} are well known as reversible and irreversible, respectively, blockers of OHC electromotility (Takehata et al., 1996). To our knowledge, however, there is no information about the effect of these agents on OHC slow motility. Thus, we incubated isolated OHCs for 3 min in extracellular solution plus salicylate (10 mM; $n=6$), Gd^{3+} (3 mM; $n=4$), or no additional drugs (Control; $n=4$), and exposed them to a 50 Hz, 8 Vpp electrical stimulus (Fig. 11 A).

As expected, both salicylate and Gd^{3+} inhibited electromotility, decreasing amplitude from 2.4 ± 0.2 % in control group to 0.4 ± 0.2 % (salicylate; $p < 0.001$) and 0.3 ± 0.3 % (Gd^{3+} ; $p < 0.001$) (Fig. 10 B). Slow motility-mediated OHC contraction (control = 1.8 ± 0.5 %), in contrast, was inhibited by Gd^{3+} (0.5 ± 0.06 %; $p < 0.04$) but unaffected by salicylate (1.3 ± 0.5 %; $p = NS$).

4. Discussion

Isolated guinea pig OHCs showed robust motile responses, with no evidence of cellular damage, when exposed to 8 Vpp electrical stimulus at frequencies ranging from 50 Hz to 4

kHz. Since cell movement in the EAEF was not constrained by any attachment of the OHCs to either the floor of the experimental chamber or the rim of a suction micropipette that could influence the mechanical response of the OHC lateral wall, the responses may be entirely attributed to the interaction of the electrical field with cellular mechanisms. Thus this traditional technique, when used in conjunction with high-speed video recording and adequate image analysis software as in the present study, may be a powerful tool to investigate the cellular and molecular mechanisms involved in OHC motility.

4.1. OHC motile responses are not significantly affected by electrode polarization

The name “electrode polarization” is applied to changes in electrical potential at the electrode-electrolyte interface induced by the passage of current (Geddes et al., 1989). Electrode polarization depends, among other parameters, on the electrolyte, the current density and the frequency, and it may change the magnitude of the electrical field generated by the electrodes (Serghei et al., 2009). We indeed found that the magnitude and strength of the electrical field generated by our experimental setup decreased when the frequency of the stimulation increased (Fig. 6 A and B). Under our experimental conditions, however, OHC motile responses were not significantly affected by this change in electrical field strength, with the cells showing similar electromotile amplitudes and slow changes in length when stimulated at the original and the corrected value of EAEF strength (Fig. 6 C). Of course, we cannot rule out a significant effect of electrode polarization on the motile responses of OHCs at frequencies above 4 kHz or with a different experimental setup. Thus, the external electrical field should be always monitored with an additional pair of electrodes to diminish the probability of errors associated with this phenomenon.

4.2. Electromotile amplitude is frequency-dependent

The frequency-dependence of electromotile amplitude was previously described in experiments using the microchamber technique (Dallos et al., 1995a; Dallos et al., 1995b; Frank et al., 1999). The conclusions of these studies, however, were conflicting. Dallos and Evans reported a pattern composed by a low-frequency asymptote below 200 Hz switching to a high-frequency asymptote above 2 kHz with zero slope, that is, with an undiminished electromotile amplitude up to the limit of their measurements at 22.5 kHz. Frank and coworkers, in contrast, described a response where the amplitude and phase responses were independent of frequency up to a cell length-dependent corner frequency, never lower than 5 kHz, after which both decreased monotonically. Our results are consistent with those from Dallos and Evans. As illustrated in Figs. 6C and 8, we found that electromotile amplitude was near constant up to 200 Hz, progressively decreased between 200 Hz and 2 kHz, and then remained near constant until the end of the experiment at 4 kHz. Interestingly, amplitude was even independent of the magnitude of electrical stimulation (8, 12 or 16 Vpp) at frequencies above 200 Hz, with changes in OHC length following cycle-by-cycle the stimulatory signal without evident differences in phase between them. These results suggest the existence of some kind of fundamental process responsible for keeping a constant value of electromotile amplitude for frequencies in the kHz range.

4.3. Electromotile amplitude increases with time of stimulation

Our results indicate that electromotile amplitude of isolated OHCs gradually increased with time, reaching the highest value after 1 s of continuous single frequency stimulation (Fig. 7) and in approximately 2 s in the case of single frequency bursts (Fig. 9). The time required to reach the plateau, however, was dependent on the length of the period between stimulations. We speculated this progressive increase in electromotile amplitude could be associated with a rearrangement of the motor proteins in the plane of the plasma membrane, a similar reorganization in the actin-spectrin membrane skeleton (which allegedly provides the vectorial component to the forces generated in the plasma membrane), or both. However, we

cannot rule out other mechanisms —like changes in membrane conductance— or even the possibility of an artifact associated with, for example, loose attachment of the cells to the glass at the bottom of the experimental chamber, although these alternative explanations are not completely consistent with the five minutes-long progressive decrease in the time required to reach the plateau detected in our experiments. Studies aimed at elucidating the source of this variation in electromotile amplitude are currently being performed in our laboratory.

4.4. Slow motility responses start milliseconds after the beginning of electrical stimulation

The stable shape and properties of actin-based structures, such as the OHC cortical cytoskeleton, result of a dynamic steady-state owing to a precise balance between actin polymerization and depolymerization (Paavilainen et al., 2004; Revenu et al., 2004). Slow OHC motility, as any cellular process powered by actin polymerization/depolymerization, depends on rapid bursts of actin filament assembly and/or disassembly at specific subcellular locations (Chesarone et al., 2009). In the case of OHCs, the subcellular location is, unmistakably, the cortical cytoskeleton.

Our results show that actin-dependent slow motile responses become detectable about 600 ms after electrical stimulation, and OHC's shorten at a rate of approximately $1\mu\text{m/s}$ (Fig. 9). These values are comparable to the fastest responses measured in vitro, which indicate that thin actin filaments polymerize and grow at rate of $2\text{--}3\mu\text{m/s}$ (Higashida et al., 2004). Thus, the speed of the responses in our experiments, together with the fact that OHC shortening requires simultaneous and cooperative work of many actin filaments all along the lateral wall, suggest that OHC's slow motility is a programmed response of the cortical cytoskeleton, and support the hypothesis that it could be a key process for the mechanism of cochlear amplification.

4.5. Is the resting potential or the voltage-electromotility function shifting during EAEF stimulation?

As depicted in Fig. 9 A, the ratio between electromotile amplitude in the elongation and contraction phases decreases with time during EAEF stimulation. This change might be associated either with a shifting of the operating point of the electromotile mechanism (a change in the electrical potential inside the cell) or a shifting of the voltage-electromotility function to the hyperpolarized direction (Santos-Sacchi et al., 1998). With the methodology used in the present study we cannot measure the electric potential in the OHC's cytoplasm, but it could be speculated that electrical stimulation and/or motile responses could activate ion channels in the plasma membrane altering the membrane resting potential. On the other hand, changes in membrane tension, perhaps associated with reorientation of plasma membrane microdomains, might induce a shift in the motility function without changes in the rest potential (Iwasa, 1993; Kakehata et al., 1995). Interestingly, our results show that changes in the ratio between electromotile amplitude in the elongation and contraction phases become apparent only after the start of slow motility, suggesting that they could be somewhat related to cytoskeletal reorganization. Clearly, more research is necessary to elucidate the underlying causes of this peculiar response.

4.6. Salicylate and Gd^{3+} affect OHC motile responses differently

Our results confirm previous reports by other authors respect to the inhibition of OHC electromotility by salicylate and Gd^{3+} , and provide evidence that Gd^{3+} , but not salicylate, interfere with OHC slow motility (Fig. 11).

The inhibitory effects of salicylate and Gd^{3+} on OHC electromotility have been known for near twenty years (Dieler et al., 1991; Kakehata et al., 1996; Santos-Sacchi, 1991; Shehata et

al., 1991; Tunstall et al., 1995). However, whereas the effect of salicylate may be removed together with the agent, Gd^{3+} -induced inhibition is irreversible. To our knowledge there is no reports about the effect of any of these agents on OHC slow motility, although it has been suggested that Gd^{3+} and other lanthanides might induce OHC elongation and increase cell stiffness (Santos-Sacchi, 1991).

Gd^{3+} is a trivalent ion with a high charge density and a similar ionic radius to Ca^{2+} . It is known as a non-selective blocker of cation channels, including some stretch-activated channels (Caldwell et al., 1998; Sachs, 2010). Gd^{3+} is able to bind lipid headgroups, increasing compression and phase separation in cell membranes (Ermakov et al., 2001); Gd^{3+} -induced pore formation in red blood cell membranes has also been reported (Cheng et al., 1999). Inside the cells, Gd^{3+} specifically displace Ca^{2+} from their binding sites on actin, affecting actin polymerization and depolymerization by creating a state (crystalline actin tube) with properties unlike those of G-actin or F-actin (Barden et al., 1978; Barden et al., 1979; Barden et al., 1980; Curmi et al., 1982; dos Remedios et al., 1980). Thus, whereas the reported effects of Gd^{3+} on OHC electromotility could be associated with a direct interaction of this lanthanide with the lipid bilayer, changing the physical properties of the membrane and indirectly affecting prestin responses, its effect on slow motility could be linked to inhibition of the actin-based mechanism located in the OHC cytoplasm. Kakehata and Santos-Sacchi (1996) reported that intracellularly applied Gd^{3+} did not affect OHC electromotility, but they did not investigate slow motility or any effect of Gd^{3+} on OHC actin-based structures. Consequently, they did not discuss the possibility that the absence of any inhibitory effect of intracellularly applied Gd^{3+} could have been associated with the sequestration of most of it by the cytoskeleton, with few ions effectively reaching the plasma membrane. This is another example of the usefulness of including slow motility studies on any evaluation of the mechanical response of OHCs.

5. Conclusions

Isolated OHCs showed strong motile responses at frequencies of up to 4 kHz when stimulated with an EAEF. OHCs followed cycle-by-cycle the electrical signal, contracting when the cuticular plate faces the cathode and elongating when it faces the anode, and with the basal pole always moving in anti-phase. In addition, electrical stimulation was followed in just milliseconds by changes in total cell length associated with slow motility responses. Whereas electromotile amplitude increased during the first 1 or 2 s of stimulation until reaching a plateau, slow motile changes started 0.6 s after the beginning of stimulation and continued for at least 3 s after its end. Cells stimulated in continuous, burst and sweeping modes showed motile responses dependent on the frequency, mode and intensity of the stimulation. Fast motility, but not slow motility, responses were affected by incubation of the cells with 10 mM salicylate; incubation with 3 mM Gd^{3+} , in contrast, affected both fast and slow motility. In addition, data collected from these studies are perfectly suited for analysis of the individual contribution of fast and slow motility to the total mechanical response of OHCs. Thus, combination of external electrical stimulation, high-speed video-recording and advanced image analysis software provides information about OHC motile responses at acoustic frequencies with an unprecedented detail, opening new areas of research in the field of OHC mechanics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

OHC	Outer hair cell
EAEF	External alternating electrical field
Vpp	Volts peak-to-peak
Gd³⁺	Gadolinium
SE	Standard error of the mean

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Highlights

- > EAEEF stimulus varied from 50 Hz to 4 kHz in continuous, burst and sweeping modes.
- > Electromotile amplitudes ranged from 0.3 % to 3.2 % of total cell length.
- > Electromotile amplitude remained constant from 2 kHz up to 4 kHz.
- > Total cell length decreased in amounts varying from 0.1 % to 4.3 %.
- > Salicylate affected electromotility but not slow motility; Gd^{3+} affected both.

Schematic diagram of the experimental setup used in the reported studies. The sketch representing an OHC is not in-scale. It was enlarged to make it visible.

Fig. 2. Stimulus protocol

Three different protocols of EAEF stimulation were used: A – continuous single frequency applied for 3 s; B – Bursted single frequency, consisting of 100 ms stimulation and 100 ms gaps, applied for 3 s, and C – Linear sweeps from 50 Hz to 4 kHz also applied for periods of 3 s.

Fig. 3. Calibration of Electrical Field Strength

A – Image of the additional electrode used to measure the electrical potential at different points between the EAEF electrodes (black shadows on the sides). B – Diagram showing typical values, expressed in mV, corresponding to an applied stimulation of 8 Vpp as a 50 Hz square wave. C – Computer-generated image of the electrical field showing isopotential lines based on the values showed in B. One 70 μ m-long OHC is represented, roughly in-scale, at the midpoint of the imaginary line connecting the center of both electrodes

Fig. 4. Resolution of the measuring system

The smallest change in OHC length directly resolved by our measurement system was estimated in 26 nm (approximately 0.04 % of OHC length). This value was susceptible to further reduction using statistical methods (Frolenkov et al., 1997).

Fig. 5. Relationship between EAEF strength and OHC motile responses

A – Electromotile amplitude (black circles) and cell length decrease (white circles) were directly proportional to EAEF strength. OHC length measurements were performed 3 s after the end of the 3 s stimulation period (4 Vpp - n=4; 8 Vpp - n=6; 12 Vpp - n=3; 16 Vpp - n=4). Error bars = SE. B – Effect of cell orientation respect to the EAEF. Solid line represents electromotile amplitude when the cuticular plate was facing the live (+/-) electrode, whereas the dashed line describes the same response when the cuticular plate was facing the ground electrode. Whereas electromotile amplitude was similar in both cases, it was a 180° difference in phase between the responses.

Fig. 6. Electrode polarization

A – Electric potential vs. frequency as measured at the midpoint of a virtual line connecting the center of stimulating electrodes using an additional pair of reference electrodes. B – EAEF strength vs. frequency as measured along the virtual line connecting the center of stimulating electrodes. C – Electromotile amplitude vs. frequency as measured using 8 Vpp electrical stimulation (black circles), and the curve corrected for electrode polarization by normalizing EAEF strength to 8 V/cm for every frequency (white circles). Curve fitting was performed using Sigma Plot 11.

Fig. 7. Single frequency continuous stimulation

A – OHC motility induced by continuous 8 Vpp, 50 Hz electrical stimulation for 3 s. Recording was performed at 1,000 fps. B – Electromotile amplitude typically increased at a rate of 0.1 % per 100 ms during the first 500 ms, and then the pace more than triplicates reaching a plateau at values around 3 % in about 1.5 s.

Fig. 8. Square wave linear sweep

A – OHC motility induced by a 50 Hz to 4 kHz square wave sweep in a 3 s period. The applied stimulation was 8 Vpp, and motility was recorded at 18,000 fps. In this particular case, electromotile amplitudes at 50 Hz and 4 kHz were, respectively, 2.0 % and 0.7 %. B – Electromotile amplitude vs. frequency at different EAEF strengths. At every value of electrical stimulation, the electromotile amplitude was near constant up to 200 Hz, progressively decreased between 200 Hz and 2 kHz, and remained near constant again between 2 and 4 kHz. Note that frequency in the abscissas axis is in log scale, whereas in panel A they are in linear scale.

Fig. 9. Single frequency burst stimulation

A – Typical motile responses in a single OHC stimulated with 50 Hz, 8 Vpp electrical bursts for 3 s. Note the gradual increase in electromotile amplitude and decrease in total cell length (slow motility). B – OHC slow motility induced by burst stimulation at frequencies of 50 Hz (n=8), 1 kHz (n=4) and 4 kHz (n=3). Cell shortening was first detected 0.6 s after the start of stimulation (arrow). C – Electromotile amplitude in the same OHCs. There is no evidence of plateau in cells stimulated at 1 kHz and 4 kHz.

Fig. 10. Simultaneous analysis of fast and slow motility components

Typical changes in shape induced by EAEF on an isolated OHCs estimated from measurements of L and A and using the equation $L=A^2/V$ (with L, A and V being the relative changes in length, section area and volume, respectively) derived by Matsumoto & Kalinec (Matsumoto et al., 2005a). Note the progressive increase of 1 % and 8 % in area and volume, respectively, after 4 s of electrical stimulation.

Fig. 11. Effects of salicylate and gadolinium on OHC motile responses

OHCs were incubated for 3 min with either 10 mM salicylate (n=6) or 3 mM Gd^{3+} (n=3) and exposed to 8 Vpp, 50 Hz electrical stimuli. Both drugs inhibited electromotility, but only Gd^{3+} affected slow motility.