Two Distinct Ca\(^{2+}\)-Dependent Signaling Pathways Regulate the Motor Output of Cochlear Outer Hair Cells

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The outer hair cells (OHCs) of the cochlea have an electromotility mechanism, based on conformational changes of voltage-sensitive “motor” proteins in the lateral plasma membrane. The translocation of electrical charges across the membrane that accompanies electromotility imparts a voltage dependency to the membrane capacitance. We used capacitance measurements to investigate whether electromotility may be influenced by different manipulations known to affect intracellular Ca\(^{2+}\) or Ca\(^{2+}\)-dependent protein phosphorylation. Application of acetylcholine (ACh) to the synaptic pole of isolated OHCs evoked a Ca\(^{2+}\)-activated apamin-sensitive outward K\(^+\) current. It also enhanced electromotility, probably because of a phosphorylation-dependent decrease of the cell’s axial stiffness. However, ACh did not change the voltage-dependent capacitance either in conventional whole-cell experiments or under perforated-patch conditions. The effects produced by the Ca\(^{2+}\)-ionophore ionomycin mimicked those produced by ACh. Hyperpolarizing shifts of the voltage dependence of capacitance and electromotility were induced by okadaic acid, a promoter of protein phosphorylation, whereas trifluoperazine and W-7, antagonists of calmodulin, caused opposite depolarizing shifts. Components of the protein phosphorylation cascade—IP\(_3\) receptors and calmodulin-dependent protein kinase type IV—were immunolocalized to the lateral wall of the OHC. Our results suggest that two different Ca\(^{2+}\)-dependent pathways may control the OHC motor output. The first pathway modulates cytoskeletal stiffness and can be activated by ACh. The second pathway shifts the voltage sensitivity of the OHC electromotility mechanism and may be activated by the release of Ca\(^{2+}\) from intracellular stores located in the proximity of the lateral plasma membrane.

Key words: sensory transduction; electromotility; voltage-dependent capacitance; cochlea; endoplasmic reticulum; patch clamp; organ of Corti

Outer hair cells (OHCs)—the sensorimotor receptors of the mammalian cochlea—elongate and shorten at acoustic frequencies when their intracellular potential is changed (for review, see Dallos, 1992; Frolenkov et al., 1998a). This unique property turns OHCs into active devices that amplify the sound-evoked mechanical responses of the organ of Corti. The ability of OHCs to change shape in a voltage-dependent manner is generally referred to as electromotility and is presumably based on voltage-driven conformational changes of densely packed putative “motor” proteins in the lateral plasma membrane (Kalinec et al., 1992). These conformational changes entail the translocation of electrical charges across the plasma membrane, observed as fast transient currents at the onset and offset of transmembrane voltage steps (Santos-Sacchi, 1991) similar to the “gating” currents recorded from voltage-dependent ion channels. However, the conformational changes of the OHC motor proteins are not associated with a net ion flow across the membrane (Santos-Sacchi and Dilger, 1988) but produce changes in the surface area of the membrane (Kalinec et al., 1992). The motor’s charge movement is sensitive to chemicals inhibiting OHC electromotility (Tunstall et al., 1995; Kakahata and Santos-Sacchi, 1996). It commonly saturates for membrane voltages below −120 mV and above +80 mV, which imparts a bell-shaped dependence to the membrane capacitance (Santos-Sacchi, 1991).

Clearly, electromotility involves a novel mechanism of force production distinct from conventional ATP-dependent, cytoskeletal-based contractile processes (Kachar et al., 1986). Nonetheless, the cylindrical shape of OHCs is maintained by a cortical cytoskeleton (Holley et al., 1992), and the elongation produced in OHCs by the Ca\(^{2+}\)-ionophore ionomycin has been attributed to a Ca\(^{2+}\)/calmodulin-dependent phosphorylation of cytoskeletal proteins (Dulon et al., 1990; Coling et al., 1998).

OHCs are the target of an efferent innervation originating in the brainstem (Warr, 1992). The principal neurotransmitter of this efferent system is acetylcholine (ACh) (Eybalin, 1993). Whole-cell recordings from isolated OHCs have provided evidence of cholinergic receptors localized around the base of the cell, where the efferent synapses are located (Housley and Ashmore, 1991). The action of ACh on OHCs requires extracellular Ca\(^{2+}\) (Blanchet et al., 1996; Evans, 1996), is accompanied by changes of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (Doi and Ohmori, 1993), and is mediated by a novel receptor, named the e\(_0\) ACh receptor (Elguyen et al., 1994). Recently Dallos et al. (1997) showed that ACh increases the electromotile responses of OHCs and attributed this effect to a decreased axial stiffness, presumably mediated by Ca\(^{2+}\)-dependent phosphorylation of unspecified cytoskeletal proteins (Szonyi et al., 1999). The molecular targets of these Ca\(^{2+}\)-mediated intracellular cascades remain difficult to identify because the OHC axial stiffness depends also on the transmembrane potential (Frolenkov et al., 1998b; He and Dallos, 1999). This suggests that the motor output of OHC can be modulated by regulatory mechanisms that target both the cytoskeleton and the membrane motor proteins.

In the present study we used capacitance measurements to investigate whether the voltage-sensitive membrane component of the OHC electromotility mechanism can be directly affected by intracellular Ca\(^{2+}\) or by Ca\(^{2+}\)-dependent signaling pathways involving protein phosphorylation.

MATERIALS AND METHODS

Cell preparation. Adult guinea pigs (200–400 gm) were killed by suffocation with carbon dioxide and decapitated. The temporal bones were removed.
from the skull and placed in modified Leibovitz cell culture medium (L-15) containing the following inorganic salts (in mM): NaCl (138), KC1 (5.3), CaCl2 (1.25), MgCl2 (1.0), Na2HPO4 (1.34), KH2PO4 (0.44), and MgSO4 (0.5). The pH was adjusted (in-mm) NaCl (142), KC1 (5.4), CaCl2 (1.3), MgCl2 (1.5), HEPES (5), tetrathylenmonium chloride (20), CsCl (20), and CoCl2 (2). The osmolarity of the extracellular solution containing CsCl was adjusted to 325 mosM with D-glucose.

To facilitate the pressure application of acetylcholine, and longer experiments involving the pressure application of acetylcholine, and longer experiments, we recorded the image of the OHC under epifluorescent microscopy (Axiovert 135, Zeiss, Jena, Germany) through a 5× objective and a 40× objective. The OHCs were maintained at room temperature (22–24°C) throughout the experiments.

Capacitance measurement. Measurements of cell capacitance were performed using the “membrane test” feature of the pCLAMP 7.0 acquisition software, which continuously delivered a test square wave of period 100 kHz using a standard laboratory interface (Digidata 1200A; Axon Instruments, Foster City, CA). Current and voltage were sampled at 100 kHz using a standard laboratory interface (Digidata 1200A; Axon Instruments, Foster City, CA). Current transients evoked by 5 mV steps. At the end of perforated-patch experiments, we recorded the image of the OHC under epifluorescent microscopy (Axiovert 135, Zeiss, Jena, Germany) through a 5× objective and a 40× objective. The OHCs were maintained at room temperature (22–24°C) throughout the experiments.

Patch-clamp recordings. As a result of the enzymatic treatment and mechanical dissociation, isolated OHCs showed different degrees of cell damage. This required careful selection of the cells before patch clamping. No report was made in the literature that allowed the comparison of potential interference contrast images of OHCs in situ with isolated ones. Therefore, we relied on the several years of experience of these laboratories to determine cell viability based on the following morphological features: uniform cylindrical shape, basal location of the nucleus, membrane birefringence, and intact stereocilia. Shorter cells (34–52 μm) were used for experiments involving the pressure application of acetylcholine, and longer cells (40–79 μm) were used for the other experiments. Pipettes for conventional and perforated-patch recordings (Horn and Marty, 1988) were filled with a modification of the pipette solution, with exchange of solution (BPS-4; ALA Scientific Instruments, Westbury, NY). OHCs were maintained at room temperature (22–24°C) throughout the patch-clamp experiments.

Patch-clamp recordings were performed using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Current and voltage were sampled at 100 kHz using a standard laboratory interface (Digidata 1200A; Axon Instruments) controlled by pCLAMP 7.0 software (Axon Instruments). The uncompensated pipette resistance was typically 3–5 MΩ when measured in the bath. The access resistance did not exceed 15 MΩ under perforated-patch conditions. Measurements of the membrane conductance were corrected off-line for the error caused by the access resistance. Junction potentials were –4.2 mV for the conventional intracellular and extracellular solution combination and –5.3 mV for the ion channel-blocking combination, as computed by the pCLAMP 7.0 software on the basis of the solution composition. These values were very similar and rather small; therefore no correction was applied to the data for liquid junction potentials.

Drug delivery. A puff pipette, prepared similarly to the patch pipette, was filled with ACh (Sigma, St. Louis, MO) or ionomycin (Calbiochem) dissolved in the extracellular solution. It was placed near the synaptic pole (Ach; Fig. 1A) or the lateral wall (ionomycin) of the OHC, and 10 kPa of pressure was applied to its back by a pneumatic injection system (PLI-100; Medical Systems Corporation, Greenvale, NY) gated under software control.

Capacitance measurement. Measurements of cell capacitance were performed using the “membrane test” feature of the pCLAMP 7.0 acquisition software, which continuously delivered a test square wave of period T = 4 msec to the cell, through the patch-clamp amplifier. This produced transient currents that decayed exponentially with time constant τ (Fig. 1B, top left). The software was designed for the simultaneous on-line measurement of τ and the linear current transients that decayed exponentially with time constant τ (Fig. 1B, top right). Therefore we relied on the several years of experience of these laboratories to determine cell viability based on the following morphological features: uniform cylindrical shape, basal location of the nucleus, membrane birefringence, and intact stereocilia. Shorter cells (34–52 μm) were used for experiments involving the pressure application of acetylcholine, and longer cells (40–79 μm) were used for the other experiments. Pipettes for conventional and perforated-patch recordings (Horn and Marty, 1988) were filled with a modification of the pipette solution, with exchange of solution (BPS-4; ALA Scientific Instruments, Westbury, NY). OHCs were maintained at room temperature (22–24°C) throughout the patch-clamp experiments.

The voltage step V elicited a whole-cell current:

\[ i = \frac{V}{R_m + R_a} \left( 1 + \frac{R_a}{R_m} \exp(-t/\tau) \right) \]

where:

\[ \tau = \frac{R_m R_a}{R_m + R_a} C_m \]

The charge delivered to the equivalent circuit by the transient current is:

\[ Q = \int_0^{T/2} V \frac{R_m}{(R_m + R_a) R_a} \exp(-t/\tau) \, dt = \frac{V C_m}{R_m} \left( 1 - \exp(-T/2 \tau) \right) \]

and the total resistance is:

\[ R_a = \frac{R_m R_a}{R_m + R_a} \]

Solving simultaneously Equations 1–3 yields:

\[ C_m = \frac{V}{\tau \frac{R_m}{R_a}} \left( 1 - \exp(-T/2 \tau) \right) \]

Because the time constant τ of the patch-clamp amplifier was typically in the range 0.1–0.3 msec at holding potentials from –50 to –70 mV, >99.8% of the current had settled within T/2 < 2 msec.

The cell capacitance during test ramps was continuously monitored at a resolution of 25 Hz, by averaging the responses to 10 positive and 10 negative consecutive test steps. The series resistance and linear capacitance compensation circuitry of the patch-clamp amplifier were not used. Instead, to determine the voltage dependence of Cm, we performed triangular voltage ramps, swelling the cell potential from Vfs = 100 mV to Vfs + 160 mV (where Vfs is the holding potential) in 6 sec (Fig. 1C). Measured values of Rm were corrected for the slope of the ramp. To test the accuracy of this experiment, we recorded the image of the OHC under epifluorescent illumination before and after breaking the perforated patch with negative pressure. Fluorescent signals were detected from the cell only after breaking the patch, indicating that the perforated patch had broken the passage of substances with a molecular weight comparable with, or larger than, that of the fluorescent probes.

The three-electrode solution contained 150 mM KC1, 0.5 mM MgCl2, 2 mM EGTA, 0.5 mM Na2HPO4, 8 mM NaH2PO4, 2 mM Mg-ATP, 2 mM Na-GTP, 0.5 mM HEPES, 5 mM Mg-ATP, 2 mM, and Na-GTP (0.2), adjusted to pH 7.4 with KOH and brought to 325 mosM with D-glucose. When using ion channel blockers in the extracellular medium, pipettes were filled with (in mM): CaCl2 (1.40), MgCl2 (2.0), EGTA (5.0), HEPES (5), Mg-ATP (2.0), and Na-GTP (0.2), adjusted to pH 7.4 with CsOH and brought to 325 mosM with D-glucose.

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with the overall surface area of the cell. However, the nonlinear voltage-dependent fraction of the cell capacitance is proportional to the area of the lateral membrane surface, where the putative motor elements are located (Huang and Santos-Sacchi, 1993). Therefore, to compare the data obtained from different cells, the nonlinear voltage-dependent capacitance was divided by the area of the lateral plasma membrane as follows:

\[ \Delta L_{\text{max}} = \frac{C_{\text{m}}(V)}{C_{\text{m}}(V_{\text{rest}})} \]

where \( C_{\text{m}}(V) \) is the specific nonlinear voltage-dependent capacitance of the lateral plasma membrane (in \( \mu \text{F/cm}^2 \)), \( C_{\text{m}}(V_{\text{rest}}) \) is the capacitance of the cell at the cell resting potential, and \( A_{\text{p}} \) gives the linear voltage-independent capacitance of the lateral plasma membrane. \( x_{\text{m}} = 1 \mu \text{F/cm}^2 \) is the specific capacitance of a lipid bilayer.

**Motility measurements.** Motility measurements were performed as described in Frolenkov et al. (1997). Briefly, OHC movements were recorded with a video camera interfacing with an inverted microscope equipped with differential interference contrast optics to an optical disk recorder (Panasonic TQ-3031F). Digitized images were analyzed off-line with the image-processing system Image 1 (Universal Imaging, West Chester, PA).

For movement quantification, a measuring rectangle ranging in length from 5 to 20 \( \mu \text{m} \) and composed of 3–15 rows of pixels was positioned across the moving edge of the cell. The average intensity profile across the edge of the cell was calculated, and the number of points in the profile was increased 10 times by cubic spline interpolation. Movement of the cell edge was calculated from the frame-to-frame shift (computed by a least-square procedure) in the interpolated intensity profiles. The sensitivity of the measurement was determined previously (Frolenkov et al., 1997). Data obtained in this way were fitted by the scaled Boltzmann function:

\[ \Delta L = \frac{L_{\text{max}}}{L_{\text{0}}} (V) = A_{\text{p}} - \frac{L_{\text{max}}}{L_{\text{0}}} \left[ 1 + \exp \left( -\frac{V - V_{\text{p}}}{W} \right) \right] \]

where \( L_{\text{0}} \) is the length of the cell at the holding potential \( V_{\text{p}} \), whereas \( \Delta L_{\text{max}} \) is the maximum voltage-dependent length change, \( V_{\text{p}} \) and \( W \) have the same meaning as in the nonlinear capacitance expression, and \( A_{\text{p}} \) is a suitable constant, such that \( \Delta L(V) = 0 \).

**Electrophysiological measurements.** Following removal of extracellular media (open circles; see Materials and Methods), data from two representative cells were fitted by the scaled derivatives of Boltzmann functions with the following parameters:

- \( C_{\text{m}} = 24.3 \mu \text{F/cm}^2 \), \( V_{\text{p}} = 22.3 \mu \text{V} \), and \( W = 24.3 \mu \text{V} \)
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\[ \Delta L(V) = \frac{L_{\text{max}}}{L_{\text{0}}} \left[ 1 + \exp \left( -\frac{V - V_{\text{p}}}{W} \right) \right] \]

Here \( L_{\text{0}} \) is the length of the cell at the holding potential \( V_{\text{p}} \), whereas \( \Delta L_{\text{max}} \) is the maximum voltage-dependent length change, \( V_{\text{p}} \) and \( W \) have the same meaning as in the nonlinear capacitance expression, and \( A_{\text{p}} \) is a suitable constant, such that \( \Delta L(V) = 0 \).

**Receptor-operated channels.** Light from a 175 W stabilized xenon arc source (Lambda DG-4; Sutter Instruments) was coupled via a liquid light guide to the epifluorescence section of an Axiomat microscope (Carl Zeiss, Jena, Germany), which was equipped with an Omega Optical XF100 filter block optimized for the Ca2+-selective dye Oregon Green 488 BAPTA-1. The illumination intensity was attenuated with a neutral density filter to avoid phototoxicity by reducing dye photo-bleaching rates to \( \leq 0.1\% \text{sec}^{-1} \). Fluorescence images were formed on a scientific grade cooled CCD sensor (Micromax 1300V; Princeton Instruments, Trenton, NJ) using an oil-immersion objective (100\( \times \); numerical aperture [NA] = 1.40; PlanApo; Carl Zeiss). The sensor’s output was binned 3 \times 3 and digitized at 12 bits/pixel to produce images that were recorded to a host personal computer controlled by the Axios Imaging Workbench 2.2 software (Axon Instruments) and analyzed off-line. For each image pixel, fluorescence signals were computed as ratios \( \Delta F = \left[ F(t) - F(0) \right] / F(0) \), where \( t \) is time. \( F(t) \) is the fluorescence after a stimulus that causes Ca2+ elevation with the cell, and \( F(0) \) is the prestimulus fluorescence computed by averaging 10–20 images.

**Immunofluorescence.** For immunofluorescence, guinea pig cochleae \( (n = 12) \) were opened and fixed in 4\% paraformaldehyde in PBS, pH 7.4, for 1 hr. Samples were permeabilized with 0.5\% Triton X-100 in PBS for 30 min, followed by overnight incubation in blocking solution (5% goat serum plus 2\% bovine serum albumin in PBS). Samples were incubated for 1 hr with 2.5 \mu g/ml affinity-purified primary antibody: the anti-Ca2+-calmodulin-dependent protein kinase IV (CaMK-IV) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-IP3 receptor antibody (Calbiochem). As a secondary antibody, we used the FITC-conjugate anti-rabbit IgG (Amersham, Piscataway, NJ). Samples were viewed with a Zeiss laser scanning confocal microscope or a Zeiss Axioskop microscope equipped with a 63\times objective (NA = 1.4). No signal was detected using the
secondary antibody alone. For the CaMK-IV labeling, we also performed an additional control, in which the primary antibody was preadsorbed for 1 hr at room temperature with an excess of the immunogen peptide (50 μg/ml), which suppressed the labeling.

RESULTS

Validation of the recording procedure

It is a common practice to block at least the most prominent K+ membrane conductance to facilitate the investigation of the electromotility-associated charge movement and/or voltage-dependent OHC capacitance (see Santos-Sacchi, 1991; Gale and Ashmore, 1997). Unfortunately, this approach cannot be applied to the study of the effects of ACh on the OHC voltage-dependent capacitance because the Ca2+-activated outward K+ current is the main indicator of the successful activation of OHC ACh receptors. Therefore, it was necessary to develop a procedure for the simultaneous measurement of \( C_m \) and cell motility (\( \Delta L \)) under conditions of varying \( R_m \). When the ratio of \( R_m \) to \( R_c \) (see Materials and Methods) was > 0.6, the error affecting our capacitance measurements was < 16% (Fig. 1B). This allowed us to determine the voltage dependence of motility and capacitance from the same voltage ramp applied to the cell membrane under whole-cell patch-clamp recording conditions (Fig. 1C). The OHC nonlinear capacitance followed the derivative of the motile response (Fig. 1D), as described previously (Santos-Sacchi, 1991). The differences between the Boltzmann parameters used to fit motility and capacitance data—the midpoint potential \( V_p \) and the potential sensitivity \( (W) \) (see Materials and Methods)—were not statistically significant at the \( p = 0.05 \) level for \( n = 14 \) control cells.

As a control, we measured also the voltage dependence of OHC capacitance in a different set of cells using intracellular and extracellular solutions designed to block the majority of ionic membrane conductances (see Materials and Methods). The values of \( V_p \), \( W \), and the maximum specific capacitance \( \chi_{\text{max}} \) were \(-18 \pm 4 \) mV, \( 33 \pm 1 \) mV, and \( 2.1 \pm 0.2 \mu \)F/cm² (\( n = 10 \)) for normal and \(-30 \pm 9 \) mV, \( 36 \pm 2 \) mV, and \( 2.0 \pm 0.1 \mu \)F/cm² (\( n = 20 \)) for blocking solutions, respectively. No statistically significant differences between the two groups were found (\( p > 0.05 \); Fig. 1E, two sample curves shown). These parameter values are in agreement with previous reports (see Santos-Sacchi, 1991). Two samples of current–voltage (\( I-V \)) relationships, obtained from each group by subjecting the membrane potential to a ramp, are plotted in the Figure 1E, inset. With blocking solutions, the whole-cell current was mainly caused by a voltage-independent leakage conductance that reversed near 0 mV.

Our measurements of \( C_m \) were robust relative to the changes of \( R_m \) not only in a model electrical circuit (Fig. 1B) but also in real cells, provided that the value of \( R_p/R_c \) was sufficiently high. Figure 2 illustrates an experiment in which the electrically evoked OHC movements partly destroyed the seal between pipette and membrane, resulting in the development of a leak. After the voltage ramps, the apparent \( R_m \) fell from \(-60 \) to 6 MΩ, changing the \( R_p/R_c \) ratio from 10 to 0.5. In spite of such dramatic changes, our system satisfactorily tracked the capacitance of the cell. Nevertheless, we were unable to measure \( C_m \) in short OHCs (<30 μm), in which a large inward current was activated at potentials more positive than \(-20 \) mV, resulting in a dramatic drop of \( R_m \) below the value of \( R_c \). Because capacitance could not be reliably measured under such unfavorable conditions, these cells have not been included in the results. This was an unfortunate limitation, because the largest ACh-evoked currents were found in such short cells from the high-frequency end of the cochlea (see also Housley and Ashmore, 1991).

Effect of ACh on the OHC voltage-dependent capacitance and electromotility

Focal applications of ACh (100 μm) to the synaptic pole of the OHC, held at approximately \( V_h = -60 \) mV (\( n = 10 \)), elicited outward currents of 50–200 pA and simultaneous 5–40% drops in \( R_m \) (Fig. 3A). The latency of this outward current was in the range of 150–250 msec (Fig. 3B), i.e., much longer than the drug delivery time that was estimated on the order of 20 msec, on the basis of similar experiments in which salicylate was applied to elicit a rapid decrease in the OHC nonlinear capacitance (Tunstall et al., 1995) (G. I. Frolov, unpublished results). In two experiments, a small inward current preceded the ACh-evoked outward current (Fig. 3C). The voltage dependence of the latter (Fig. 3D) had a characteristic N shape (Blanchet et al., 1996; Evans, 1996). These data agree with the view that ACh activates a small inward current, partly carried by Ca2+, which in turn triggers a large Ca2+-activated outward K+ current (Evans, 1996). Application of apamin (1 μm) to the bath suppressed the ACh-evoked outward current (data not shown), indicating that it was carried through small-conductance Ca2+-activated K+ channels (Blatz and Magleby, 1986; Yamamoto et al., 1997).

In spite of the prominent ACh-induced current responses, we observed virtually no ACh-induced changes in the OHC voltage-dependent membrane capacitance (Fig. 3A,E,F). However, simultaneous measurements of the length changes of the same cell showed a significant ACh-induced increase of the electromotile responses (Fig. 4). In a group of cells showing a well preserved cilindrical cell body (\( n = 4 \), which we took as an indication of normal turgor condition, the electromotile responses increased by 2–26% after ACh, without statistically significant changes (at \( p = 0.05 \) level) in the peak value of \( C_m(V) \). After ACh, the midpoint of the voltage sensitivity of the membrane capacitance shifted slightly toward more hyperpolarized values (\( \Delta V_p = -3.9 \pm 0.8 \) mV; \( p < 0.05 \)). The ACh-induced stationary elongation of the cells of this group (\( \Delta L = 2.4 \pm 3.0\% \)) was not statistically significant (\( p = 0.48 \)).

Cell turgor (intracellular pressure) is an important factor in the control of OHC electromotility (Shehata et al., 1991; Chertoff and Brownell, 1994) and nonlinear capacitance (Iwasa, 1993; Kakehata and Santos-Sacchi, 1995). Therefore, it was possible that the increase of electromotility after ACh depended on turgor increase, with turgor and ACh effects canceling each other at the level of the nonlinear capacitance. To exclude this possibility, we tested the effect of ACh on OHCs (\( n = 4 \)) whose normal turgor had been removed by applying negative pressure to the patch pipette (see Kakehata and Santos-Sacchi, 1996; Santos-Sacchi and Huang, 1998). ACh did not change the maximal voltage-dependent capacitance of these collapsed OHCs but shifted slightly the peak of \( C_m(V) \) (\( \Delta V_p = -3.3 \pm 1.2 \) mV; \( p < 0.05 \)).
Acetylcholine does not affect OHC capacitance. A. Monitoring cell parameters before, during, and after two consecutive applications of ACh (100 μM; 20 sec; open horizontal bars). From top to bottom, the following parameters were measured: I, whole-cell current; R, access resistance; Rm, membrane resistance; and Cm, membrane capacitance. Trace deflections are caused by the delivery of triangular voltage ramps to the cell (see Fig. 1C; ramps are numbered 1–7). B. Sample of the current response to ACh (100 μM; 1 sec) from a different cell, shown on a faster time scale to reveal a barely noticeable inward current preceding the large outward current. C. Current–voltage I–V relationship without ACh (control; closed squares; average of the data from ramps 1, 3, 4, 6, and 7) and during the first (ACh1; open circles) and the second (ACh2; open triangles) application of ACh in A. D, ACh-sensitive fraction of the I–V curve, obtained by subtraction of the whole-cell current during ACh application from the mean of the whole-cell currents before and after ACh (data from C). E, F, Insensitivity of the capacitance–voltage Cm(V) relationship to ACh, measured before (control; closed squares), during (open squares), and after (washout; closed triangles) the first (E) and the second (F) application of ACh (ACh1, ACh2 in A). Cm(V) relationships were obtained from ramps 1–6 indicated in parentheses. Data were fitted by the scaled derivatives of Boltzmann functions. All data points were obtained at Rm/R < 0.7; therefore the estimated error of Cm measurements was < 2 pF.

Figure 4. Functional decoupling of electromotility and nonlinear capacitance after ACh application. Capacitance–voltage relationships Cm(V) (top) and the voltage dependence of electromotile responses ΔL(V) (bottom), measured before (control; closed squares), during (ACh; open circles), and after (washout; closed triangles) ACh application. Data are from the same experiment shown in Figure 3, A and C–F. Capacitance data were fitted by the scaled derivative of Boltzmann functions with the following parameters: Cmax = 15 pF, C0 = 16 pF, V50 = 55 mV, and W = 21 mV (control); Cmax = 15 pF, C0 = 16 pF, V50 = 50 mV, and W = 21 mV (ACh); and Cmax = 16 pF, C0 = 15 pF, V50 = 57 mV, and W = 22 mV (washout). Motility data were fitted by the Boltzmann functions with the following parameters: ΔLmax = 4.5% V50 = 36 mV, and W = 25 mV (control); ΔLmax = 5.6%, V50 = 52 mV, and W = 23 mV (ACh); and ΔLmax = 4.9%, V50 = 38 mV, and W = 24 mV (washout).

Effect of ionomycin

To determine whether the elevation of [Ca2+]i affects the OHC nonlinear capacitance, we applied the Ca2+ ionophore ionomycin. This drug is known to induce a generalized, transient increase of [Ca2+]i by making the plasma membrane, as well as the membranes of intracellular Ca2+ stores, permeable to Ca2+ (Liu and Hermann, 1978; Smith et al., 1989).

Puff applications of ionomycin (25 μM; 20 sec) produced dramatic increases of [Ca2+]i in OHCs (ΔF/F = 84 ± 28%); n = 10), widespread along the whole-cell body (Fig. 5A–C). The [Ca2+]i increase was accompanied by a voltage-independent elongation of the cell (Fig. 5C, middle) amounting, on average, to ΔL/L0 = 4.6 ± 0.9% (n = 10; p < 0.001). In 70% of the cells this elongation was terminated by the loss of cell turgor, either temporary or permanent. Usually, the [Ca2+]i, initially elevated by ionomycin showed a very slow decline, and it did not return to the baseline within 10–20 min. Further applications of ionomycin to the same cell produced additional step-like increases of [Ca2+]i up to the saturating level of the fluorescent indicator. Saturation was commonly reached after the second or third application. Only data from the first applications are reported here.

Similar to ACh, ionomycin was able to evoke a transient outward current in 50% of the cells (range, 15–320 pA; n = 6) at Vh = −50 mV (Fig. 5C, bottom). The voltage dependence of this current had an N shape (Fig. 5E, inset) similar to that of the ACh-induced outward current (compare Fig. 3D). The simplest explanation is that ionomycin evoked Ca2+-activated K+ current. At the concentration of 10 μM, ionomycin evoked only an outward current in three out of seven cells tested. In the remainder of the cells, no current response was detected, although an increase of [Ca2+]i was always observed. At the higher concentration (25 μM), in four out of nine cells the outward current was followed by an inward current with a reversal potential close to zero (data not shown). Such inward current was generally found in cells with a high basal level of [Ca2+]i, as judged by the resting fluorescent level of the cell and by the relatively small difference between resting and saturating fluorescence. Ca2+-activated nonselective cation channels, possibly underlying the observed inward currents, have been described in OHCs (Van den Abbeele et al., 1996). The nature of this second current was not investigated further.

Usually, we did not observe any substantial changes of OHC voltage-dependent capacitance after ionomycin application, except at a relatively high drug concentration (50 μM) or with repeated applications of the drug to cells whose resting [Ca2+]i level was initially already high (data not shown). In cells with low resting...
Figure 5. Ionophore-mediated elevation of intracellular free Ca\(^{2+}\) concentration increases the OHC resting length, enhances electromotile responses, but does not affect nonlinear capacitance. A. Fluorescent image of an OHC filled with Oregon Green 488 BAPTA-1 (50 \(\mu\)M) through the patch pipette. B. The same cell shown in A 1 min after the application of 25 \(\mu\)M ionomycin. Scale bar, 10 \(\mu\)m. C. Time course of fluorescence changes (top), resting length (middle), and whole-cell current (bottom) after application of ionomycin to the cell shown in A and B. Fluorescence intensity was computed by averaging pixel values throughout the cell body. Deflections on the current and length records are caused by the delivery of triangular voltage ramps (numbered 1–4) to the cell. A closed horizontal bar at the bottom of the panel indicates the timing of the drug application. D. Membrane capacitance–voltage \(C_m(V)\) relationships before (control; ramp 1; closed squares) and after (ramps 2, 3, 4; open symbols) application of ionomycin, obtained during the correspondingly numbered voltage ramps in C. Data were fitted by the scaled derivatives of the Boltzmann function. E. Electromotility responses of a different OHC before application of ionomycin (Control; closed squares) and after recovery from ionomycin-induced turgor loss (Ionomycin, open circles; 2 min after drug application). Data were fitted by Boltzmann functions. Inset, A sample of the current–voltage relationship of the ionomycin-sensitive fraction of the whole-cell current.

[Ca\(^{2+}\)]\(_i\), ionomycin produced virtually no changes of the cell nonlinear capacitance, even at a concentration of 25 \(\mu\)M (Fig. 5D).

Three cells were observed in bright field to investigate electromotile responses before and after the application of ionomycin (25 \(\mu\)M). In all three cases the maximum voltage-activated motile responses of the OHCs increased (Fig. 5E) by 0.73, 0.63, and 1.24% of the cell length \(0.87 \pm 0.19\%\) (mean \(\pm\) SE); \(p < 0.05\).

Effect of ACh and ionomycin on OHCs under perforated-patch conditions

Metabotropic effects of ACh may be significantly compromised under conventional whole-cell patch-clamp recording conditions because of washout of intracellular constituents during the first minutes after breaching the membrane patch (Horn and Marty, 1988). Therefore, we investigated the effects of ACh and ionomycin on the cell’s membrane capacitance under perforated-patch conditions, i.e., when the membrane patch at the pipette tip was not physically broken but was made permeable to small ions with nystatin (Horn and Marty, 1988). Under these conditions, we managed to measure reliably the OHC capacitance only in the two experiments shown in Figure 6. Neither ACh (Fig. 6A; 100 \(\mu\)M; 20 sec) nor ionomycin (Fig. 6B; 25 \(\mu\)M; 20 sec) produced measurable changes of \(C_m\) in these experiments.

Modulation of the operating range of OHC electromotility by protein phosphorylation

It has been suggested that the effect of ACh on OHC electromotility is mediated by a Ca\(^{2+}\)-dependent phosphorylation of cytoskeletal proteins (Dallos et al., 1997). We tested whether phosphorylation affects also the voltage sensor of the OHC motor by investigating the effects of inhibitors and promoters of protein phosphorylation on \(\chi_m(V)\). Cells were preincubated at 37°C in okadaic acid, a powerful inhibitor of protein phosphatase-1 and -2A that promote phosphorylation of a wide range of proteins in vivo (Haystead et al., 1989), and the specific calmodulin inhibitors trifluoperazine and W-7. After incubation, the cytoplasmic morphology of the cells in the dish did not change visibly. A significant number of OHCs remained viable according to our selection criteria (see Materials and Methods). After incubation in okadaic acid (1 \(\mu\)M; 30–60 min), \(\chi_m(V)\) shifted in the hyperpolarized direction (Fig. 7, top). Incubation for 30–60 min with trifluoperazine (30 \(\mu\)M) and W-7 (150 \(\mu\)M) shifted \(\chi_m(V)\) in the opposite direction (Fig. 7, top). The voltage dependence of the electromotile responses, \(\Delta L(V)\), was affected similarly (Fig. 7, middle). The effects of these reagents on \(\chi_m(V)\) did not depend on intracellular pressure because they were reproducible both in artificially collapsed cells and in cells with apparently normal turgor. In artificially collapsed OHCs we observed the following values of the potential at the peak of \(\chi_m(V)\): \(-37.7 \pm 3.1\) mV (control; \(n = 9\)), \(-56.8 \pm 5.2\) mV (okadaic acid; \(n = 4\)), \(-2.2 \pm 1.9\) mV (trifluoperazine; \(n = 3\)), and \(-0.9 \pm 1.2\) mV (W-7; \(n = 3\)). Parameters of \(\chi_m(V)\) and \(\Delta L(V)\) relationships for OHCs with apparently normal turgor are shown in Table 1. Comparing the effects of these reagents (Fig. 7) with those produced by ACh (Fig. 3) indicates that the shift of \(\chi_m(V)\) induced by ACh is only \(\sim 5\%\) of the maximal range covered by phosphorylation.

The reagents tested did not change significantly the maximal nonlinear capacitance \(\chi_{max}\), the maximal motile response \(\Delta L_{max}\), or the voltage sensitivity \(W\) of capacitance and electromotility, with the exception of okadaic acid that changed the voltage sensitivity \(W\) of \(\chi_m(V)\) (Table 1). However, they increased the zero-current voltage (Fig. 7, bottom), probably because of the disruption of mechanisms responsible for maintaining the intracellular potential (Hasin and Barry, 1984).

It is well known that isolated OHCs are depolarized (Ashmore, 1987). Immediately after achieving the whole-cell configuration, the zero-current potential \(V_z\) is rarely more negative than \(-30\) mV and gradually shifts to more hyperpolarized values over 3–5 min as potassium in the patch pipette equilibrates with the cell interior. This explains the difference in the values of \(V_z\) in Figures 3C and 7, bottom. The latter were obtained within 60 sec after breaching the patch of membrane under the recording pipette to minimize the dialysis of the intracellular constituents with the pipette solution. The \(-mV\) shift in \(V_z\) reported for long OHCs in the presence of okadaic acid (Jagger and Ashmore, 1999) is not comparable with data in Figure 7 simply because the latter were not obtained in steady-state conditions.
and in the absence of ion channel blockers (Fig. 1E) argues against the possibility that the effects of phosphorylation are indirect effects on ion channels that in turn affect the motors. Calcium currents in OHCs are extremely small, and none of the several \( Ca^{2+} \) channel blockers we tested (Frolenkov et al., 1998a) had any effect on OHC electromotility. Therefore, it seems unlikely that the observed effects may be caused by modification of calcium influx produced by the phosphorylation/dephosphorylation of calcium channels. These effects are also not related to the changes of cell turgor, because they were present in artificially collapsed cells as well as in cells with apparently normal turgor.

We have measured the voltage dependence of the nonlinear membrane capacitance \( C_m(V) \) and length change \( \Delta L(V) \) in isolated OHCs without blocking ion channels. In these cells, most ion channels are localized to a fraction of the plasma membrane near the synaptic pole (Santos-Sacchi et al., 1997), where their density is several fold lower than the density of the putative motor proteins in the basolateral membrane [up to \( 6000/\mu m^2 \) (Frolenkov et al., 1998a)]. On the basis of our own measurement of the total motor’s charge density movement:

\[
\int_{-n}^{+n} \chi_m(V) dV
\]

**Immunohistochemical localization of \( Ca^{2+} \) release channels and CaMK-IV**

\( Ca^{2+} \)/calmodulin-dependent protein phosphorylation often involves the release of \( Ca^{2+} \) from \( IP_3 \)-gated intracellular stores (Berridge, 1993). To determine whether the key elements of this pathway colocalize with the electromotile apparatus, we immunolabeled whole-mount preparations of the organ of Corti with fluorescent antibodies raised against \( IP_3 \) receptors (Fig. 8, left) and CaMK-IV (Fig. 8, right). Labeling for both the \( IP_3 \) receptors and CaMK-IV was found to be concentrated at the cell cortex, along the OHC lateral wall between the nucleus and the cuticular plate. The lateral plasma membrane of the OHC contains the putative molecular motors (Dallos et al., 1991; Kalinec et al., 1992) and is underlined by a cortical cytoskeleton adjacent to layers of endoplasmic reticulum named lateral cisternae (Holley et al., 1992). The thicker pattern of labeling was observed for the \( IP_3 \) receptor localization (Fig. 8, left), suggesting that it is associated with the lateral cisternae. The thinner labeling observed for the CaMK-IV (Fig. 8, right) suggests association with the cortical cytoskeleton or the plasma membrane. Some punctuated labeling was also observed below the cuticular plate and at the synaptic pole of the OHC, for both the \( IP_3 \) receptor and the CaMK-IV.

**DISCUSSION**

Our results show, for the first time, that the voltage sensitivity of the OHC electromotile mechanism can be modulated by reagents affecting \( Ca^{2+} \)/calmodulin-dependent phosphorylation. Inhibition of protein phosphorylation produces a depolarizing shift of voltage sensitivity, whereas activation leads to a hyperpolarizing shift (Fig. 7). The invariance of the capacitance measurement in the presence of voltage-sensitive calcium current affects \( Ca^{2+} \) currents in OHCs are extremely small, and none of the several \( Ca^{2+} \) channel blockers we tested (Frolenkov et al., 1998a) had any effect on OHC electromotility. Therefore, it seems unlikely that the observed effects may be caused by modification of calcium influx produced by the phosphorylation/dephosphorylation of calcium channels. These effects are also not related to the changes of cell turgor, because they were present in artificially collapsed cells as well as in cells with apparently normal turgor.

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\]
ionophore ionomycin is followed by cell elongation promoted by a Ca\(^{2+}\)/calmodulin-dependent pathway (Dulon et al., 1990; Coling et al., 1998). In our experiments both ACh and ionomycin evoked outward currents (Figs. 3, 4, 6) and under whole-cell patch-clamp recording conditions. However, there are strong indications for the existence of functional intracellular Ca\(^{2+}\) stores in close proximity to the OHC electromotile machinery.

In conclusion, our data suggest that the OHC motor output may be affected by two Ca\(^{2+}\)-dependent pathways. One pathway targets the proteins of the cortical cytoskeleton, altering the global axial stiffness of the cell. The other pathway targets the putative membrane motors, shifting its operating range. Our results show that the natural ligands ACh (Figs. 3, 4, 6) and ATP (Mammano et al., 1999) do not cause changes in the voltage sensitivity of the membrane motors in isolated OHCs maintained at room temperature and under whole-cell patch-clamp recording conditions. However, there are strong indications for the existence of functional intracellular Ca\(^{2+}\) stores in close proximity to the OHC electromotile machinery. Release of Ca\(^{2+}\) from such stores may potentially modulate the function of the OHC motors very effectively. It remains to be determined what sort of physiological stimuli may be responsible for the activation of these putative Ca\(^{2+}\)-release-dependent cascades.

### Table 1. Parameters of \(\chi_{\text{max}}(V)\) and \(\Delta L(V)\) relationships for the control group of OHCs and the cells preincubated with okadaic acid (1 \(\mu M\)), trifluoperazine (30 \(\mu M\)), and W-7 (150 \(\mu M\))

<table>
<thead>
<tr>
<th></th>
<th>Capacitance measurements</th>
<th>Electromotility measurements</th>
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<tbody>
<tr>
<td></td>
<td>(\chi_{\text{max}}) ((\mu F/cm^2))</td>
<td>(C_0) (pF)</td>
</tr>
<tr>
<td>Control ((n = 14))</td>
<td>1.81 ± 0.10</td>
<td>22.9 ± 1.5</td>
</tr>
<tr>
<td>Okadaic acid ((n = 6))</td>
<td>1.82 ± 0.10(^N)</td>
<td>20.4 ± 1.1(N)</td>
</tr>
<tr>
<td>Trifluoperazine ((n = 6))</td>
<td>1.63 ± 0.07(N)</td>
<td>22.9 ± 1.9(N)</td>
</tr>
<tr>
<td>W-7 ((n = 7))</td>
<td>1.75 ± 0.31(N)</td>
<td>25.9 ± 3.4(N)</td>
</tr>
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</table>

Average values and the corresponding SEs are shown. To minimize the potential adverse effects of dialyzing the intracellular constituents with the pipette solution, measurements were taken within 60 sec after establishing whole-cell recording conditions. Statistical significance of the difference between values of any parameter in control and treated OHCs is designated as follows: N, no statistical significance \((p > 0.05)\); *\(p < 0.01\); **\(p < 0.001\).

Using immunofluorescence, we determined, in agreement with others (Koyama et al., 1999), that some key elements required to activate a Ca\(^{2+}\)-dependent protein phosphorylation cascade, i.e., IP\(_3\) receptors and CaMK-IV, are present along the OHC lateral wall (Fig. 8) where the putative molecular motors of the OHC are localized (Dallos et al., 1991; Kalinec et al., 1992). Like the cortical lattice in erythrocytes, the filamentous network of OHC cytoskeletal proteins seems to be anchored to the plasma membrane by periodic protein pillars \(\sim 25\) nm long (Raphael and Wroblewski, 1986). Normally, the relatively stiff circumferential filaments may restrain large changes in cell diameter, whereas the elastic cross-links offer less resistance. The cortical lattice is a highly orthotropic structure because its resultant circumferential stiffness modulus is approximately one order of magnitude larger than the axial one (Tolomeo et al., 1996). Consequently, a major function of the cytoskeleton would be to direct electrically driven shape changes along the longitudinal axis of the cell (Tolomeo et al., 1996). A system of flattened, membrane-bound intracellular compartments, the subsurface cisternae (Engstrom, 1958), is found near the cytoskeletal lattice. Closely related is the synaptic cisterna, located at the basal (synaptic) pole of the cell. Together with the cytoskeletal lattice and plasma membrane, they form a complex structure, the cell cortex (Holley et al., 1992). The preferential distribution of Ca\(^{2+}\)-ATPase near the innermost layer of the cisternae, in strict apposition to linearly arranged mitochondria, supports a role for these structures as intracellular Ca\(^{2+}\) stores (Schulte, 1993), a conclusion compatible with our present immunofluorescence data. Release of Ca\(^{2+}\) from these putative stores may activate biochemical cascades that modulate the cell’s axial stiffness and the voltage sensitivity of the plasma membrane motors.

In conclusion, our data suggest that the OHC motor output may be affected by two Ca\(^{2+}\)-dependent pathways. One pathway targets the proteins of the cortical cytoskeleton, altering the global axial stiffness of the cell. The other pathway targets the putative membrane motors, shifting its operating range. Our results show that the natural ligands ACh (Figs. 3, 4, 6) and ATP (Mammano et al., 1999) do not cause changes in the voltage sensitivity of the membrane motors in isolated OHCs maintained at room temperature and under whole-cell patch-clamp recording conditions. However, there are strong indications for the existence of functional intracellular Ca\(^{2+}\) stores in close proximity to the OHC electromotile machinery. Release of Ca\(^{2+}\) from such stores may potentially modulate the function of the OHC motors very effectively. It remains to be determined what sort of physiological stimuli may be responsible for the activation of these putative Ca\(^{2+}\)-release-dependent cascades.

### REFERENCES


