ROCK-dependent and ROCK-independent Control of Cochlear Outer Hair Cell Electromotility

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Running Title: Homeostatic regulation of OHC electromotility

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Summary

Outer hair cell electromotility is crucial for the proper function of the cochlear amplifier, the active process that enhances sensitivity and frequency discrimination of the mammalian ear. Previous work has suggested a role for Rho GTPases in the regulation of outer hair cell electromotility, though the signaling pathways mediated by these enzymes remains to be established. Here we have investigated the cellular and molecular mechanisms underlying the homeostatic regulation of the electromotile response of guinea pig outer hair cells. Our findings defined a ROCK-mediated signaling cascade that continuously modulates outer hair cell electromotility by selectively targeting the cytoskeleton. A distinct, ROCK-independent pathway functions as a fast resetting mechanism for this system. Neither pathway affects the function of prestin, the unique molecular motor of outer hair cells. These results extend our understanding of a basic mechanism of both normal human hearing and deafness, revealing the key role of the cytoskeleton in the regulation of outer hair cell electromotility and suggesting ROCK as a molecular target for modulating the function of the cochlear amplifier.
Introduction

Cochlear outer hair cells (OHCs) undergo reversible changes in length when electrically stimulated. This electromotile response results from a membrane-based force generator mechanism associated with conformational changes and rearrangement of a voltage-sensitive integral membrane protein (1). The OHC motor protein has been recently identified as a novel anion transporter (SLC26A5-prestin) (2), with voltage sensitivity conferred by the intracellular anions chloride and bicarbonate (3) (reviewed by (4)). The vectorial component to the forces generated in the OHC plasma membrane is provided by the cortical cytoskeleton, an actin-spectrin meshwork placed immediately underneath the plasma membrane and connected to it through thousands of 25 nm-long, rod-like structures (pillars) (5,6).

It has been recently demonstrated that OHC electromotility is necessary for the correct work of the cochlear amplifier, the active process that enhances sensitivity and frequency discrimination of the mammalian ear (7). Cochlear amplification is maximal at low signal levels (~0-5 dB SPL), decreases proportionally to the increase of signal intensity, and becomes inhibited for sounds of about 80 dB (4,8). This “automatic gain control” is crucial for extending the dynamic range of the auditory system, since it provides the cochlea with the ability to amplify very faint sounds and process the loudest without suffering permanent damage (9). The direct association between cochlear amplification and OHC electromotility suggests that this gain-control may be provided by the modulation of the OHC electromotile response.

OHCs must undergo continuous mechanical changes to optimize the sensitivity and frequency selectivity of the cochlea (10-13). We dubbed “mechanical homeostasis” to the process of
constant adjustment of control parameters that automatically bring OHCs near the optimal working point for each condition. The cochlea, however, may be challenged by twelve orders of magnitude variations in sound intensity in fractions of a second. Therefore, in addition to the homeostatic mechanism of continued regulation, OHCs may require a “reset” switch for fast inhibition of signal amplification in order to protect the cochlea from sudden bursts of high intensity noise. While little is known about the nature of the homeostatic control, a strong inhibitory effect on the gain of the cochlear amplifier has been associated with acetylcholine (ACh) released by efferent terminals innervating OHCs (14-16).

Recent experimental evidence suggests a role for Rho GTPases in the regulation of OHC electromotility (16). However, the molecular and cellular mechanisms controlled by these enzymes remain to be determined. We have focused our attention on RhoA and its downstream targets ROCK, known to regulate dynamic reorganization of cytoskeletal proteins (17), and adducin, a cytoskeletal protein that promotes actin-spectrin binding and caps the fast-growing ends of actin filaments (18,19). In this study, we show that the cellular mechanism of homeostatic control of OHC electromotility involves Rho-mediated cytoskeletal changes without affecting the prestin motors. In addition, we demonstrate that the molecular machinery underlying this phenomenon requires the activation of ROCK-dependent and ROCK independent pathways. These results extend our understanding of the mechanisms by which Rho GTPases regulate OHC electromotility, reveal a key role for the cytoskeleton in this phenomenon, and suggest that ROCK may serve as a molecular target for modulating the function of the cochlear amplifier.
Experimental Procedures

**Immunolabeling:** Guinea pigs (200-300g) were euthanized following procedures and protocols approved by the Institutional Animal Care and Use Committee (IACUC). For plastic sections, guinea pig cochleas were fixed in 4% paraformaldehyde (Electron Microscopy Science, Ft.Washington, PA), decalcified in 8% EDTA for 4 weeks, and embedded in celloidin following standard procedures. Sections were labeled with biotinylated secondary antibodies that react with alkaline-phosphatase-conjugated streptavidin molecules (DAKO LSAB+System AP, DAKO Corp., Carpinteria, CA). For confocal observation, whole-mount preparations and isolated OHCs were processed as described previously (16). Anti-ROCK-I (C-19), anti-ROCK-II (C-20), anti-Adducin α (C-15), anti-p-adducin<sup>Thr445</sup>, and anti-p-adducin<sup>Ser726</sup> (Santa Cruz Biotechnologies, Santa Cruz, CA) were used as primary antibodies. Blocking peptides were used in pre-adsorption (negative control) experiments. Samples were observed with Zeiss microscopes Axiovert 135 and laser confocal LSM-410 with a C-Apo 40X (NA=1.2) objective.

**Western blot.** For Western blot analyses, total cell homogenates from cochlea, lung and brain of guinea pigs were homogenized and lysed in a 50 mM Tris buffer solution, pH 7.4, containing NP-40, 1%; EDTA, 2 mM; NaCl, 100 mM; vanadate, 1 mM; 10 µl/ml of 0.1M PMSF; and 3 µl/ml of 10 mg/ml aprotinin. Samples were then mixed with the loading buffer (SDS, 0.04 g; bromophenol blue, 0.002 g; β-mercaptoethanol, 1 ml; sucrose, 4 g; and stacking buffer for 10 ml), boiled for 5 minutes, centrifuged and the supernatant stored at -20°C (all drugs from Sigma, St. Louis, MO). Cochlear and positive control samples were separated by SDS-PAGE gels (30 µg protein/lane), transferred to nitrocellulose membranes and incubated with the primary antibodies. The reaction was detected by enhanced chemiluminiscence using a peroxidase-
labeled secondary antibody (Amersham Biosciences, UK). Competition studies were performed with the antibodies and blocking peptides mentioned above.

Adducin phosphorylation: Guinea pig cochleas (n=18) were opened in Leibowitz L-15 (Gibco, Gaithersburg, MD), and the spiral exposed by removing the bony shell. Six samples were pre-incubated for 30-min with the ROCK inhibitor Y-27632 (10µM. Upstate Biotechnology, Lake Placid, NY), which is known to be incorporated into cells by a carrier-mediated facilitated diffusion (20). Next, ACh (Sigma, St. Louis, MO) was added to six samples, three of those pre-incubated with Y-27632 and three untreated, at 100 µM final concentration. Simultaneously, LPA (Sigma, St. Louis, MO) was added to other six samples, three pre-incubated with Y-27632 and three untreated, at 10 µM final concentration. After ten minutes, all the treated samples plus six without any treatment (control) were fixed in 3% paraformaldehyde and 0.1% glutaraldehyde and labeled as previously described using either anti-\(\text{p-adducin}^{\text{Thr445}}\) or anti-\(\text{p-adducin}^{\text{Ser726}}\) as primary antibodies. In similar experiments, additional guinea pig cochleas were incubated with ACh and LPA by periods of 1, 2, 4, 6 and 8 minutes.

Electromotility measurements: OHCs were isolated by microdissection, suspended in Leibowitz L-15 media in a perfusion chamber on an Axiovert 135 inverted microscope stage, and patch clamped as previously described (16). Patch pipettes were filled with internal perfusion buffer (KF, 120mM; KCl, 20 mM; MgCl\(_2\), 2 mM; EGTA, 0.5mM; Mg-ATP, 2mM; Na-GTP, 0.1mM; HEPES, 10 mM) adjusted with Trizma Base to pH 7.4 (Control), or with the internal perfusion buffer plus GST (100 µg/ml. Calbiochem, San Diego, CA), C3 (100 µg/ml. Cytoskeleton, Denver, CO), Y-27632 (10 µM. Upstate Biotechnology, Lake Placid, NY), dnRac1 and dnCdc42
(100 µg/ml. Kindly provided by Dr. J. Silvio Gutkind, NIDCR-NIH) or combinations of these compounds at identical concentrations.

After being patch-clamped, cells were permitted to stabilize in their new mechanical conditions for 8 to 10 minutes. In those experiments not involving ACh, cells were electrically stimulated with bursts of three depolarization (+50 mV)/hyperpolarization (-145 mV) cycles (3Hz) to elicit electromotility. The cell response was recorded in video and analyzed off-line. In those experiments involving ACh, Leibowitz L-15 (negative control) and ACh (100 µM), were delivered to the basolateral wall of the cells (~0.15 µl/sec) using a computer-controlled perfusion system (DAD-12, ALA Scientific Instr., Westbury, NY) as previously described (16). The electrical and structural integrity of every OHC was continuously evaluated through the whole experiment. The osmolarity of every solution used in these experiments was controlled and adjusted with glucose to 300±2 mOsm with a µOsmette 5004 freezing-point osmometer (Precision Systems Inc. Natick, MA). Experiments were fully recorded on videotape, and analyzed off-line. Changes in electromotile amplitude were measured with a resolution better than 0.1 µm as described elsewhere (21). A total of 245 cells were included in the experiments.

**Non-linear capacitance measurements**: Membrane capacitance was evaluated during electromotility experiments, and before and after ACh perfusion. We followed the transient analysis of currents protocol originally developed by Santos-Sacchi and coworkers (22). Briefly, OHCs were electrically stimulated with 15 mV stair steps from a holding potential of -145 mV to +50 mV. Capacitance function was fitted to the first derivative of a two-state Boltzmann function relating non-linear charge to membrane voltage (23). Since membrane stress may affect
capacitance measurements, special care was dedicated to diminish potential effects of uncontrolled intracellular pressure changes during evaluation of capacitance and electromotility.

Data was statistically analyzed with ANOVA (analysis of variance) techniques by using the software StatView 4.1 and SuperAnova (Abacus, Berkeley, CA). Statistical significance was set at $P \leq 0.05$. 
Results

Expression of ROCK and adducin in the guinea pig cochlea

There are two closely related isoforms of ROCK, ROCK I (a.k.a. p160 ROCK and ROK β) and ROCK II (a.k.a Rho kinase, Rho-associated kinase and ROK α) (17). ROCK I is expressed at high levels in heart, kidney, skeletal muscle, pancreas, lung and liver but it is nearly absent in brain; ROCK II, in contrast, is abundantly expressed in brain and weakly in the lung (17). In the present study, we find that both isoforms are expressed in the guinea pig cochlea at levels that appear to be higher for ROCK I than for ROCK II (Fig. 1). Confocal microscopy of isolated cells demonstrate that both ROCK I and ROCK II immunolocalize primarily at the OHC cortex (Fig. 2). This localization is consistent with the previously reported distribution of RhoA in these cells (16). Antibodies against α-adducin, a marker for the actin-spectrin cytoskeleton and a target of ROCK, showed that this protein is also expressed in guinea pig cochlea (Fig. 1), and labeled OHCs with a pattern similar to ROCK I and ROCK II (Fig. 2). Thus, these results suggest that similarly to its upstream activator and its downstream target, ROCK kinases localize primarily either immediately adjacent to or at the areas involved in OHC cell electromotility.

Homeostatic regulation of OHC electromotility

Given its abundant expression in OHCs, we hypothesized that ROCK inhibition might result in changes in the mechanical properties of these cells and consequently, in their electromotile response. To test this hypothesis, we inhibited the function of ROCK and RhoA in isolated OHCs using either pharmacological or molecular approaches and investigated their effect on cells’ electromotility. To inhibit RhoA-mediated signaling pathways we used C3 exoenzyme from Clostridium botulinum, which ADP-ribosylates RhoA (but not Rac and Cdc42) at Asn41
thereby blocking RhoA-mediated signals (24). ROCK activity was controlled with the synthetic compound Y-27632, which inhibits both ROCK-I and ROCK-II with high specificity by competing with ATP for binding to the kinases (20).

We investigated the electromotile response of OHCs to different concentrations of either C3 or Y-27632 (Fig. 3). Both C3 and Y-27632 affected the electromotile response of isolated guinea pig OHCs. For instance, Fig. 3a shows that OHC’s electromotile amplitude decreased progressively up to 70% of its control value (70.7±5.7%. P≤0.01) as C3 concentration was increased up to 100 µg/ml. In contrast, a concentration of 100 µM Y-27632 decreased electromotile amplitude up to 30% of its control value (31.1±11.4%. P≤0.001. Fig. 3b). Cells receiving 100 µM Y-27632, however, showed signals of deterioration and yielded more variable results (Fig. 3b). The cellular damage observed in this experiment likely reflects a toxic effect of the drug rather than the pharmacological effect that is usually observed at lower concentrations. Therefore, we used concentrations of 100 µg/ml for C3 and 10 µM for Y-27632 in all the subsequent experiments.

To address whether C3 and Y-27632 work cooperatively and/or synergistically, we subsequently measured electromotile amplitude in OHCs co-perfused with these two compounds. Figure 4a shows that while the electromotile amplitude of non-treated (Control) cells was 3.5±0.1% of the total cell length in these experiments, C3 (100 µg/ml) and Y-27632 (10 µM) decreased it to 2.6±0.2% (P≤0.01) and 2.7±0.2% (P≤0.01), respectively. Co-perfusion of C3 and Y-27632 decreased electromotility amplitude to 2.8±0.2%, a value that was not significantly different from that observed with either C3 or Y-27632 alone (Fig. 4a). These results suggest that
RhoA and ROCK work “in serie”, and that a RhoA/ROCK-mediated signaling cascade might be antagonizing a parallel pathway that decreases OHC electromotility.

To investigate whether these results might be associated with the existence of a parallel pathway mediated by other small GTPases, we used previously characterized dominant-negative constructs of Rac1 (dnRac1) and Cdc42 (dnCdc42) (16). As shown in Fig. 4b, electromotile amplitude did not change when OHCs were co-perfused with dnRac1+C3 or dnRac1+C3+Y-27632. Replacement of dnRac1 by dnCdc42, in contrast, resulted in a significant reduction in OHC electromotile amplitude. Although these results appear to suggest a role for Cdc42 in the electromotile response of OHCs, comparison of Figs. 4a and 4b provides a different interpretation. Co-perfusion with dnRac1 indeed abolished the effect of C3 and C3+Y-27632, returning the amplitude of OHC electromotile response to control values (from 2.6±0.2 to 3.4±0.1% for C3, P≤0.01, and from 2.7±0.2 to 3.6±0.2% for C3+Y-27632, P≤0.01). In contrast, dnCdc42 did not change significantly the response of OHCs to C3 or C3+Y-27632 (from 2.6±0.2% to 3.0±0.1%, and from 2.8±0.2 to 2.6±0.2%, respectively). These results indicate that Rac1, but not Cdc42, might be associated with the pathway antagonized by the RhoA/ROCK-mediated signaling cascade.

The ACh-activated signaling pathway

Subsequently, we asked whether the RhoA/ROCK-mediated pathway could be the same ACh-activated pathway previously reported as regulating OHC electromotility (16). We found that external stimulation with ACh did not change the electromotile response of isolated OHCs internally perfused with C3. While C3 alone decreased electromotile amplitude from 3.5% to
2.6% of the total cell length, ACh stimulation left amplitude statistically unchanged (2.5±0.1%. Fig. 5). The electromotile amplitude of OHCs internally perfused with the ROCK inhibitor Y-27632, in contrast, was increased about 22% by ACh, bringing the response back to control values (from 2.7±0.2 to 3.4±0.1% of the total cell length. P≤0.025). Importantly, this ACh-induced significant increase was abolished by co-perfusion of Y-27632 and C3 (2.7±0.2. Fig. 5). Altogether, these results suggested that the signaling cascade activated by ACh would be mediated by RhoA but not by ROCK. Therefore, it would not be the same pathway involved in the homeostatic control of OHC electromotility.

Next, we wondered whether adducin could help us to confirm that the ACh-activated pathway is indeed ROCK-independent. It has been suggested that phosphorylation of adducin by ROCK at Thr 445 (p-adducin$^{Thr445}$) facilitates the recruitment of spectrin to F-actin and promotes the assembly of the actin-spectrin cytoskeleton beneath the plasma membrane (25). On the other hand, PKC-mediated phosphorylation of adducin at Ser 726 (p-adducin$^{Ser726}$) would inhibit the recruitment of spectrin to F-actin, inducing the disassembly of the actin-spectrin meshwork (25). Using antibodies that label phosphorylated forms of this protein, we found a pool of both p-adducin$^{Thr445}$ and p-adducin$^{Ser726}$ in untreated, isolated OHCs (Fig. 6a,b). Importantly, pre-incubation of guinea pig cochleas with Y-27632 for 30 minutes inhibited adducin phosphorylation both at Thr 445 and Ser 726 (Figs. 6c,d). To investigate whether adducin was involved in the ACh-activated pathway, guinea pig cochleas were incubated with ACh for 1, 2, 4, 6, 8, and 10 minutes respectively, fixed and labeled with anti-p-adducin$^{Thr445}$ and anti-p-adducin$^{Ser726}$. We found that ACh did not increase significantly the amount of labeled adducin (Figs. 6e-h), confirming that ACh activates a ROCK-independent pathway. As a positive control,
we replaced ACh with LPA. LPA, activates a signaling pathway involving G_{α13}, RhoGEF-115, and RhoA (Neves et al, 2002). Subsequently, RhoA, activates ROCK and PKC resulting in the phosphorylation of adducin at Ser. 726 (via PKC) and Threonine 445 and 480 (via ROCK). In contrast to ACh, just two minutes incubation with LPA was enough to greatly increase the amount of p-adducin$^{\text{Thr}445}$ and p-adducin$^{\text{Ser}726}$ (Fig. 6i,j), and both LPA-induced responses were abolished by pre-incubation with Y-27632 (Figs. 6k,l).

Interestingly, we found stronger labeling of p-adducin$^{\text{Thr}445}$ in the hair bundle than in the OHC body (Fig. 6a, insert). Labeling of p-adducin$^{\text{Ser}726}$, in contrast, was homogeneous (Fig. 6b). Since the actin-spectrin cytoskeleton is not a major component of the OHC’s hair bundle, these results suggested that adducin might be functioning there as an actin-capping protein (18), modulating the renewal of actin in the stereocilia (26).

**Regulation of OHC electromotility is a prestin-independent process**

Finally, we measured voltage-dependent non-linear capacitance ($C_m$) in isolated OHCs to explore whether the observed variations in electromotile amplitude could be associated with changes in the performance of prestin. $C_m$ results from the movement of electrical charges across the plasma membrane associated with conformational changes in the motor proteins (4). In addition, a positional shift of its voltage peak value ($V_{pkC_m}$) indicates the existence of indirect effects (i.e., changes in cell turgor) that modify the operating point of the motors (27-30). In a first series of experiments, we found that up to 30 minutes’ incubation of isolated OHCs with Y-27632 did not induce significant changes either in $C_m$ or $V_{pkC_m}$, (results not shown). Next, we measured $C_m$ before and after external stimulation with ACh (Fig. 7). In agreement with a recent
report by Frolenkov and co-workers (31), we did not detect any significant effect of ACh on untreated OHCs (Fig. 4b). Similar results were obtained in OHCs internally perfused with Y-27632 (Fig. 7c), in spite of the significant increase in electromotile amplitude induced by ACh in these cells (Fig. 7c). Thus, we conclude that neither the pathways involved in mechanical homeostasis nor the one activated by ACh affect the performance of the motor proteins in a direct manner. An indirect effect on prestin mediated by changes on OHC turgor is also unlikely because we did not observe any shift in $V_{phCm}$ (Figs. 7b,c). Since cell turgor is sensitive to ion flux, changes in surface potential and changes in membrane tension, a side effect of the treatment on these parameters may also be ruled out.
DISCUSSION

In this study, we provide evidence emphasizing the importance of prestin-independent processes in the control of OHC electromotility. We define pathways that link distinct Rho-GTPases to the remodeling of the OHC cytoskeleton. A ROCK-dependent pathway would be continuously adjusting the electromotile response of the OHC in order to optimize the sensitivity and frequency selectivity of the cochlea. A ROCK-independent pathway, activated by ACh, functions as a reset system to bypass the homeostatic mechanism and returns the cell to a mechanical equilibrium. These findings are integrated in the tentative model illustrated in Fig. 8. This model predicts that the gain of the OHC electromotile response is controlled by the RhoA/ROCK regulated inhibition of a potent, constitutively active, amplitude-decreasing signaling cascade mediated by Rac1. The complete inhibition of the Rac1-mediated pathway, on the other hand, would result in the largest electromotile response compatible with both the structural and functional parameters of the force generator mechanism and the mechanical load working on the system. This safety feature minimizes the risk of stereocilia damage and deafness associated with uncontrolled increase in the electromotility amplitude, thus protecting the auditory system against acoustic trauma.

OHC mechanical homeostasis

The cochlear amplifier increases the dynamic range of the cochlea by enhancing the vibration of the basilar membrane. Continuous operation, responding and adapting to a fluctuating input, demands a feedback mechanism for automatic (homeostatic) control (10-13). Demonstration that OHC electromotility is necessary to explain cochlear amplification (7) leads naturally to
associate homeostatic regulation of the cochlear amplifier with a parallel homeostatic regulation of the OHCs’ electromotile response.

The results summarized in Figs. 3 and 4a clearly indicate that C3 and Y-27632, inhibitors of RhoA and ROCK respectively, modify the electromotile response of untreated, isolated OHCs. Note that neither the C3 nor the Y-27632 dose-response curves show a clear saturation in their effect on the OHC electromotile response (Fig. 3). Inhibition achieved with higher doses of these agents, however, may be unreliable, as suggested by the toxic effect of 100 µM Y-27632 described above. Nonetheless, inhibition of about 40% in OHC electromotility such as reported here significantly support the participation of RhoA and ROCK in the regulation of this phenomenon. Since C3 and Y-27632, used either alone or jointly, decreased OHC electromotile amplitude, we hypothesized that this effect could be mediated by the inhibition of a RhoA/ROCK-mediated signaling cascade regulating an amplitude-decreasing pathway (Fig. 8). The experiments with dnRac1 and dnCdc42 described in Fig. 4b support this hypothesis, and suggest that this amplitude-decreasing pathway would be mediated by Rac1 but not Cdc42.

What could be the structural target for these signaling pathways mediated by members of the Rho family of small GTPases that control OHC electromotility? Three potential molecular mechanisms may be envisioned. First, Rho-mediated signals could be directly targeting the membrane-embedded molecular motors. The conformational changes in the individual motor molecules or the number of them participating in the response, for instance, could be selectively altered. Second, Rho-mediated signals could be inducing cytoskeletal changes that modify the OHC mechanical response only by changing the mechanical load on the molecular motors.
Finally, these potential Rho-mediated cytoskeletal changes could be influencing the performance of the membrane-embedded motors either directly or indirectly through changes in other parameters like membrane tension. Our measurements of voltage-dependent nonlinear membrane capacitance, both during homeostatic regulation and when activated by ACh, clearly imply the cytoskeleton as the only target for the RhoA-mediated signaling cascade. These results also suggest that a significant indirect effect on the performance of the membrane-embedded molecular motors, mediated by changes in membrane tension or ion flux for instance, is unlikely.

Adducin experiments provided indirect support to the hypothesis that the cytoskeleton is the essential structure involved in the homeostatic regulation of OHC electromotility. ROCK activation by RhoA induces $p$-adducin$^{\text{Thr}445}$ and $p$-adducin$^{\text{Thr}480}$, promoting the recruitment of spectrin to actin filaments, the formation of the actin-spectrin cytoskeleton, and its connection to the plasma membrane via ERM proteins (25). PKC and PKA, in contrast, inhibit this activity and induce the disassembly of the actin-spectrin cytoskeleton by generating $p$-adducin$^{\text{Ser}726}$ (18,32). The finding in untreated OHCs of $p$-adducin$^{\text{Thr}445}$ and $p$-adducin$^{\text{Ser}726}$ suggests that the OHC cortical cytoskeleton is dynamically regulated, with continuous assembly and disassembly of the actin-spectrin meshwork. Together, the experiments reported here indicate that the activation of a RhoA/ROCK-mediated pathway induces both adducin phosphorylation and regulation of OHC electromotility. This data also suggest that future research aimed at defining the role of adducin and adducin-associated proteins in the biology of OHCs will contribute significantly to the better understanding of OHC electromotility.
In addition to promoting actin-spectrin association, adducin is known to act as an actin-capping protein (18). Adducin’s actin capping, like spectrin recruitment activities, is activated by ROCK-mediated phosphorylation and inhibited by PKC-mediated phosphorylation (32). Kachar and co-workers described recently that the actin filaments forming the core of hair cell stereocilia are continuously remodeled (26). Complete remodeling is achieved every 48 hours by addition of actin monomers to the stereocilium tips. The strong labeling of \( p\)-adducin\(^{Thr445}\) in the hair bundle of untreated OHCs (Figs. 6a, insert) suggests that actin renewal could be another homeostatic process regulated by a ROCK-mediated pathway involving adducin.

**Regulation of OHC electromotility by ACh**

The dynamic range of the mammalian cochlea is about 120 dB, from the softest to the loudest sounds the cochlea can detect without permanent damage. That means that the cochlea can withstand sound pressures that are one million times greater in amplitude, and therefore with \(10^{12}\) more energy, than sounds at the threshold of detection (9). It survives because the mechanical sensitivity and amplification are progressively reduced as the sound level increases. A self-regulated homeostatic response may be insufficient, however, to protect the cochlea from sudden bursts of high energy noise. ACh released by the efferent terminals innervating OHCs could be required to disengage this “automatic gain control” and rapidly inhibit amplification in response to changes in the auditory input that may result in cochlear damage.

The results summarized in Fig. 5 suggest that ACh influences OHC electromotility by activating a signaling pathway mediated by RhoA but not by ROCK. Adducin experiments supported this hypothesis by showing that ACh did not increases ROCK-mediated
phosphorylation at Thr 445 (Figs. 6e-h). Therefore, this ACh-activated pathway is different from those involved in OHC mechanical homeostasis. Since ACh reverse the decrease in electromotility amplitude induced by ROCK inhibition, we envision this ACh-activated pathway as a reset system able to move the cell quickly back to a mechanical steady state, ensuring the inhibition of the cochlear amplifier when necessary to protect the auditory system from acoustic trauma.

If ACh would always be increasing OHC electromotile amplitude, why did we not detect any ACh-induced electromotile increase in untreated, isolated OHCs? According to the proposed model (Fig. 8), the upper value for the ACh-induced increase in OHC electromotile amplitude should be equal to the biggest response compatible with structural and functional parameters of the force generator mechanism and the external and internal mechanical load working on it. Therefore, an isolated OHC (no external mechanical load) in complete mechanical equilibrium with the medium (lowest internal load) would respond to a given stimulus with the biggest possible electromotile amplitude for that condition. Amplitude in the control group, in consequence, would be near the upper limit for the electromotile response in our experimental conditions, and they could not be further increased by ACh. Thus, the ACh effect would be evident only in isolated cells away from mechanical equilibrium or unable to reach it by a particular treatment. This was the response observed, for instance, in OHCs treated with the ROCK inhibitor Y-27632, where the ACh effect was to bring amplitude back to control values (Fig. 5).
CONCLUSIONS

We provided evidence that the small GTPase RhoA and its downstream target ROCK are crucial for the mechanical homeostasis and the regulation of the electromotile response of cochlear OHCs. Our results support a model where a RhoA/ROCK-mediated signaling cascade would be able to inhibit a Rac1-mediated parallel pathway that decreases OHC electromotile amplitude. Higher levels of inhibition would result in bigger OHC electromotile amplitude, providing a safe and reliable automatic control for the cochlear amplifier. In this model, ACh should be able to inhibit the cochlear amplifier by resetting OHC electromotile amplitude to values corresponding to mechanical equilibrium. OHC electromotility regulation would be associated with cytoskeleton reorganization, without changes on the performance of the membrane-embedded molecular motors. These results are an important step toward the elucidation of the mechanisms that regulate OHC electromotility and cochlear amplification.
ACKNOWLEDGEMENTS

This work was supported in part by NIDCD/NIH grant DC05220 to F.K. and NIDCD/NIH grant DC05335 and a grant from S. Mark Taper Foundation to G.K. RU was supported by NIH grants DK52913, DK56620, and the Lustgarten Foundation.
REFERENCES


FIGURE LEGENDS

Fig. 1: ROCK I, ROCK II and α-adducin are expressed in guinea pig cochlea. (a) – Western blot analysis of cochlear proteins incubated with antibodies against ROCK I, ROCK II and α-adducin (+) and the same antibodies pre-adsorbed with the corresponding blocking peptides (-) as a negative control. (b) – As a positive control, antibodies were tested on samples of lung (ROCK I) and brain (ROCK II and α-adducin).

Fig. 2: Immunolocalization of ROCK I, ROCK II and α-adducin in guinea pig cochlea. Labeling of plastic-embedded guinea pig cochleas with anti-ROCK I (a), anti-ROCK II (b) and anti-adducin (c) suggested abundant expression of these proteins in OHCs (arrows). Confocal images of isolated OHCs (d-f) indicated that ROCK I, ROCK II and adducin localized primarily at the cell cortex. As a negative control, isolated OHCs were incubated with antibodies pre-adsorbed with their corresponding blocking peptides (g-i). Scale bars, 50 µm (a-c) and 10 µm (d-i).

Fig. 3: C3 and Y-27632 decrease OHC electromotile amplitude in a dose-dependent manner. (a) - A concentration of 100 µg/ml of C3 exoenzyme, a RhoA inhibitor from Clostridium botulinum, significantly decreases OHC electromotile amplitude to about 70% of its control value. (b) – 10 µM of Y-27632 a significant decrease in electromotile amplitude to about 60 % of the control value. Higher concentrations had an even stronger inhibitory effect, but cellular integrity was compromised. **=P≤0.01, ***=P≤0.001.
Fig. 4: OHC electromotility might be regulated by two different signaling pathways, one mediated by RhoA and ROCK and the other by Rac1. (a) - C3 (RhoA inhibitor) and Y-27632 (ROCK inhibitor) induced a similar decrease in OHC electromotile amplitude when used either separately or in combination. (b) - A dominant-negative Rac1 construct, dnRac1, abolished the decrease in OHC’s electromotile amplitude induced by C3 and Y-27632 though a dnCdc42 had no effect (compare with Fig. 4a). **=P≤0.01, ***=P≤0.001.

Fig. 5: ACh regulates the electromotile response of OHCs through a RhoA-dependent, ROCK-independent pathway. While ACh did not affect the electromotile response of isolated OHCs internally perfused with C3, it reverted to normal values the electromotile amplitude of those treated with the ROCK inhibitor Y-27632. **=P≤0.01

Fig. 6: Adducin is involved in the ROCK-dependent pathway, but not in the ROCK-independent pathway activated by ACh, modulating OHC electromotility. Confocal images of whole-mount preparations of guinea pig organ of Corti showing mid-cell optical sections of the three rows of OHCs. A pool of phosphorylated adducin was found in OHCs of untreated guinea pig cochleas (a,b). Adducin phosphorylation was significantly reduced by pre-incubation with Y-27632 (c,d) but unaffected by ACh (e-h), indicating that ACh did not activate ROCK. LPA, in contrast, significantly increased adducin phosphorylation (i,j), and this response was inhibited by Y-27632 (k,l). Labeling of p-adducin^{Thr445} (but not p-adducin^{Ser726}) was stronger in the stereocilia bundle than in the body of untreated OHCs (6a, insert). While Y-27632 practically abolished labeling of phosphorylated adducin everywhere, LPA treatment appeared to saturate the response
rendering cells with similar labeling both at the stereocilia bundle and the cell body. For details, see text. Scale bars, 20 µm (a-I) and 5 µm (inserts).

**Fig. 7:** ACh does not affect OHC voltage-dependent membrane capacitance (Cm). (a) - With the holding potential at -70 mV, whole cell current was recorded with the voltage-ramp approach from -145 to 50 mV. The current was evaluated at steady state. From the recorded data, an I-V (current-voltage) function was plotted. (b) - Cm was similar when measured in untreated cells before (control) and after application of ACh. (c) - ACh did not change Cm in OHCs internally perfused with the ROCK inhibitor Y-27632.

**Fig. 8:** Model for ROCK-dependent and ROCK-independent regulation of OHC electromotility. A Rac1-mediated, amplitude-decreasing signaling cascade would be modulated by both a RhoA/ROCK pathway and a RhoA-mediated, ROCK independent pathway activated by ACh (gray box). While the RhoA/ROCK-mediated pathway would be permanently activated (mechanical homeostasis), the ACh-activated pathway could function as a fast resetting mechanism for the OHC’s electromotile response.
Figure 1
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
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J. Biol. Chem. published online July 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301668200

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