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

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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 (Kalinec, F., Zhang, M., Urrutia, R., and Kalinec, G. (2000) J. Biol. Chem. 275, 28000–28005) has suggested a role for Rho GTPases in the regulation of outer hair cell electromotility, although the signaling pathways mediated by these enzymes remain 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 define 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 the mechanisms by which 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 that caps the fast-growing ends of actin filaments (18, 19). In this study, we show that the cellular mechanism of continued regulation, OHCs may require a “reset” switch for fast inhibition of signal amplification to protect the cochlea from sudden bursts of high intensity noise. Although 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 that 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 provided 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 sound pressure level), decreases proportionally to increases in signal intensity, and becomes inhibited at sounds of −80 db (4, 8). This “automatic gain control” is crucial for extending the dynamic range of the auditory system because it provides the cochlea with the ability to amplify very faint sounds and to 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 used the term “mechanical homeostasis” for the process of constant adjustment of control parameters that automatically bring OHCs near the optimal working point for each condition. However, the cochlea may be challenged by variations of 12 orders of magnitude in sound intensity in fractions of 1 s. Therefore, in addition to the homeostatic mechanism of continued regulation, OHCs may require a “reset” switch for fast inhibition of signal amplification to protect the cochlea from sudden bursts of high intensity noise. Although 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).

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 (Ref. 3; reviewed in Ref. 4). The vectorial component of the forces generated in the OHC plasma membrane is

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¶ The abbreviations used are: OHCs, outer hair cells; ACh, acetylcholine; LPA, lysophosphatidic acid; dn, dominant-negative.

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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 (200–300 g) were euthanized following the procedures and protocols approved by the Institutional Animal Care and Use Committee. For plastic sections, guinea pig cochleae were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA), decalcified in 8% EDTA for 4 weeks, and embedded in celluloid following standard procedures. Sections were labeled with biotinylated secondary antibodies that react with alkali phosphate-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-α-adducin (C-15), anti-phospho-Thr445 adducin, and anti-phospho-Ser726 adducin (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. Blocking peptides were used in pre-adsorption (negative control) experiments. Blocking experiments were performed with the anti-phospho-Ser726 adducin (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies. Western blotting were performed at 1:20 objective. Samples were observed with Zeiss Axiosvert 135 and LSM-410 laser confocal microscopes with a ×40 C-Apo (numerical aperture of 1.2) objective.

**Western Blotting—**For Western blot analyses, total cell homogenates from guinea pig cochlea, lung, and brain were homogenized and lysed in 50 mM Tris buffer solution (pH 7.4) containing 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, 1 mM vanadate, 0.5 mM phenylmethylsulfonyl fluoride (10 μM), and 10 mg/ml aprotinin (3 μM). Samples were then mixed with loading buffer (0.04 g of SDS, 0.002 g of bromphenol blue, 1 ml of β-mercaptoethanol, 4 g of sucrose, and 10 ml of stacking buffer (all from Sigma)), boiled for 5 min and centrifuged. The supernatant was stored at −20 °C. Cochlear and positive control samples were separated by SDS-PAGE (30 μg of protein/lane), transferred to nitrocellulose membranes, and incubated with the primary antibodies. The reaction was detected by enhanced chemiluminescence using a peroxidase-labeled secondary antibody (Amersham Biosciences, Buckinghamshire, UK). Competition studies were performed with the antibodies and blocking peptides mentioned above.

**Adducin Phosphorylation—**Guinea pig cochleae (n = 18) were opened in Leibovitz L-15 medium (Invitrogen), and the spiral was exposed by removing the bony shell. Six samples were preincubated for 30 min with the ROCK inhibitor Y-27632 (10 μM; Upstate Biotechnology, Inc., Lake Placid, NY), which is known to be incorporated into cells by carrier-mediated facilitated diffusion (20). Next, ACh (Sigma) was added to six samples (three preincubated with Y-27632 and three untreated) at a final concentration of 100 μM. Samples were then mixed with loading buffer (0.04 g of SDS, 0.002 g of bromphenol blue, 1 ml of β-mercaptoethanol, 4 g of sucrose, and 10 ml of stacking buffer (all from Sigma)), boiled for 5 min and centrifuged. The supernatant was stored at −20 °C. Cochlear and positive control samples were separated by SDS-PAGE (30 μg of protein/lane), transferred to nitrocellulose membranes, and incubated with the primary antibodies. The reaction was detected by enhanced chemiluminescence using a peroxidase-labeled secondary antibody (Amersham Biosciences, Buckinghamshire, UK). Competition studies were performed with the antibodies and blocking peptides mentioned above.

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After being patch-clamped, cells were permitted to stabilize under their new mechanical conditions for 8–10 min. In those experiments not involving ACh, cells were electrically stimulated with bursts of three depolarization (+50 mV)/hypoperpolarization (−145 mV) cycles (3 Hz) to elicit electromotility. The cell response was recorded on videotape and analyzed off line. In those experiments involving ACh, Leibovitz L-15 medium (control) or ACh (100 μM) were delivered to the basolateral wall of the cells (−0.15 μl/s) using a computer-controlled perfusion system (DAD-12, ALA Scientific Instruments, Westbury, NY) as previously described (16). The electrical and structural integrity of each OHC was continuously evaluated through the whole experiment.

**RESULTS**

**Expression of ROCK and Adducin in the Guinea Pig Cochlea—**There are two closely related isoforms of ROCK, ROCK I (p160ROCK and ROK-β) and ROCK II (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. In contrast, ROCK II is abundantly expressed in brain and weakly in lung (17). In this study, we found 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 demonstrated 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 that of ROCK I and ROCK II (Fig. 2). Thus, these results suggest that, similar to their upstream activator and downstream target, ROCK kinases localize primarily either immediately adjacent to or at the areas involved in OHC electromotility.

**Homeostatic Regulation of OHC Electromotility—**Given the abundant expression of ROCK 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 cell electromotility. To inhibit RhoA-mediated signaling pathways, we used C3 exoenzyme from Clostridium botulinum, which ADP-ribosylates RhoA (but not Rac and Cdc42) at Asn113,
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. 3 (upper panel) shows that OHC electromotile amplitude decreased progressively up to 70% of the control value (70.7 ± 6.7%; *p < 0.01) as the C3 concentration was increased up to 100 μM. In contrast, 100 μM Y-27632 decreased electromotile amplitude up to 30% of the control value (31.1 ± 11.4%; **p < 0.001) (Fig. 3, lower panel). However, cells receiving 100 μM Y-27632 showed signals of deterioration and yielded more variable results (Fig. 3, lower panel). 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 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. Fig. 4a shows that whereas the electromotile amplitude of untreated (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 electromotile 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 series” 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. In contrast, replacement of dnRac1...
Fig. 5. ACh regulates the electromotile response of OHCs through a RhoA-dependent, ROCK-independent pathway. Although ACh did not affect the electromotile response of isolated OHCs internally perfused with C3, it reverted the electromotile amplitude of those treated with the ROCK inhibitor Y-27632 to normal values. N.S., not significant; **, \( p < 0.01 \).

with dnCdc42 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, Fig. 4 (compare a and b) provides a different interpretation. Co-perfusion with dnRac1 indeed abolished the effect of C3 and C3 + Y-27632, returning the amplitude of the 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 significantly change 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.

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. Whereas C3 alone decreased electromotile amplitude from 3.5 to 2.6% of the total cell length, ACh stimulation left the amplitude statistically unchanged (2.5 ± 0.1%) (Fig. 5). In contrast, the electromotile amplitude of OHCs internally perfused with the ROCK inhibitor Y-27632 was increased −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 suggest 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 Thr445 (phospho-Thr445 adducin) 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, protein kinase C-mediated phosphorylation of adducin at Ser726 (phospho-Ser726 adducin) 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 phospho-Thr445 adducin and phospho-Ser726 adducin in untreated isolated OHCs (Fig. 6, a and b). Importantly, preincubation of guinea pig cochleae with Y-27632 for 30 min inhibited adducin phosphorylation at both Thr445 and Ser726 (Fig. 6, c and d). To investigate whether adducin is involved in the ACh-activated pathway, guinea pig cochleae were incubated with ACh for 1, 2, 4, 6, 8, and 10 min, fixed, and labeled with anti-phospho-Thr445 adducin and anti-phospho-Ser726 adducin antibodies. We found that ACh did not significantly increase the amount of labeled adducin (Fig. 6, e–h), confirming that ACh activates a ROCK-independent pathway. As a positive control, we replaced ACh with LPA. LPA activates a signaling pathway involving Gα12, RhoGEF-115, and RhoA (34). Subsequently, RhoA activates ROCK and protein kinase C, resulting in the phosphorylation of adducin at Ser726 (via protein kinase C) and at Thr445 and Thr460 (via ROCK). In contrast to ACh, just 2 min of incubation with LPA was enough to greatly increase the amount of phospho-Thr445 adducin and phospho-Ser726 adducin (Fig. 6, i and j), and both LPA-induced responses were abolished by preincubation with Y-27632 (Fig. 6, k and l).

Interestingly, we found stronger labeling of phospho-Thr445 adducin in the hair bundle than in the OHC body (Fig. 6a, inset). In contrast, labeling of phospho-Ser726 adducin was homogeneous (Fig. 6b). Because the actin-spectrin cytoskeleton is not a major component of the OHC hair bundle, these results suggest 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 nonlinear 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 the voltage peak value \( \left(V_{pk}C_m\right) \) indicates the existence of indirect effects \( (i.e. \) changes in cell turgor) that modify the operating point of the motor \( \left(27–30\right) \). In a first series of experiments, we found that up to 30 min of incubation of isolated OHCs with Y-27632 did not induce significant changes in either \( C_m \) or \( V_{pk}C_m \) (data not shown). Next, we measured \( C_m \) before and after external stimulation with ACh (Fig. 7). In agreement with a recent report by Frolenkov et al. (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 despite 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 affects the performance of the motor proteins in a direct manner. An indirect effect on prestin mediated by changes in OHC turgor is also unlikely because we did not observe any shift in \( V_{pk}C_m \) (Fig. 7, b and c). Because cell turgor is sensitive to ion flux and to changes in surface potential and membrane tension, a side effect of the treatment on these parameters may also be ruled out.

DISCUSSION

In this study, we have provided evidence emphasizing the importance of prestin-independent processes in the control of OHC electromotility. We defined 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 to optimize the sensitivity and frequency selectivity of the cochlea. A ROCK-indepen-
dent 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. On the other hand, the complete inhibition of the Rac1-mediated pathway 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 stereocilium damage and deafness associated with an uncontrolled increase in electromotile 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 the association of homeostatic regulation of the cochlear amplifier with the parallel homeostatic regulation of the OHC 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 the C3 and Y-27632 dose-response curves do not show a clear saturation in their effect on the OHC electromotile response (Fig. 3). Inhibition achieved with higher doses of these agents may be unreliable, however, as suggested by the toxic effect of 100 μM Y-27632 described above. Nonetheless, inhibition of ~40% of OHC electromotility such as reported here significantly supports the participation of RhoA and ROCK in the regulation of this phenomenon. Because 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 such as membrane tension. Our measurements of voltage-dependent nonlinear membrane capacitance, both during homeostatic regulation and when activated by ACh, clearly implicate 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 motors either directly or indirectly through changes in other parameters such as membrane tension 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 phospho-Thr^{445} adducin and phospho-Thr^{480} adducin, promoting the recruitment of spectrin to actin filaments, the formation of the actin-spectrin cytoskeleton, and its connection to the plasma membrane via ERM.
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(ezrin/radixin/moesin-protein family) proteins (25). In contrast, protein kinases C and A inhibit this activity and induce the disassembly of the actin-spectrin cytoskeleton by generating phospho-Ser256 adducin (18, 32). The finding in untreated OHCs of phospho-Thr445 adducin and phospho-Ser256 adducin 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. These 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). Like spectrin recruitment activities, adducin actin capping is activated by ROCK-mediated phosphorylation and is inhibited by protein kinase C-mediated phosphorylation (32). Kachar and co-workers (26) described recently that the actin filaments forming the core of hair cell stereocilia are continuously remodeled. Complete remodeling is achieved every 48 h by addition of actin monomers to the stereocilium tips. The strong labeling of phospho-Thr445 adducin in the hair bundle of untreated OHCs (Fig. 6a, inset) 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 _–120 db_ from the softest to the loudest sounds the cochlea can detect without permanent damage. This 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 to 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 increase ROCK-mediated phosphorylation at Thr445 (Fig. 6, e–h). Therefore, this ACh-activated pathway is different from those involved in OHC mechanical homeostasis. Because ACh reverses the decrease in electromotile 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 increase 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. In consequence, the amplitude in the control group would be near the upper limit for the electromotile response under our experimental conditions, and it could not be further increased by ACh. Thus, the ACh effect would be evident only in isolated cells not in 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, for which the ACh effect was to bring the amplitude back to control values (Fig. 5).

Conclusions—We have provided evidence that the small GTPase RhoA and its downstream target ROCK are crucial for the mechanical homeostasis and regulation of the electromotile response of cochlear OHCs. Our results support a model in which 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 in 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.

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