RHO GTPASES MEDIATE THE REGULATION OF COCHLEAR OUTER
HAIR CELL MOTILITY BY ACETYLCHOLINE

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Running Title: Rho GTPases in the regulation of OHC motility

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SUMMARY

Outer hair cells are the mechanical effectors of the cochlear amplifier, an active process that improves the sensitivity and frequency discrimination of the mammalian ear. In vivo, the gain of the cochlear amplifier is regulated by the efferent neurotransmitter acetylcholine through the modulation of outer hair cell motility. Little is known, however, regarding the molecular mechanisms activated by acetylcholine. In this study, intracellular signaling pathways involving the small GTPases RhoA, Rac1 and Cdc42 have been identified as regulators of outer hair cell motility. Changes in cell length (slow motility) and in the amplitude of electrically induced movement (fast motility) were measured in isolated outer hair cells patch-clamped in whole-cell mode, internally perfused through the patch pipet with different inhibitors and activators of these small GTPases while being externally stimulated with acetylcholine. We found that acetylcholine induces outer hair cell shortening and a simultaneous increase in the amplitude of fast motility through Rac1 and Cdc42 activation. In contrast, a RhoA- and Rac1-mediated signaling pathway induces outer hair cell elongation and decreases fast motility amplitude. These two opposing processes provide the basis for a regulatory mechanism of outer hair cell motility.
INTRODUCTION

Inside the mammalian inner ear, the mechanical stimulus provided by sound is amplified up to 100 times by a mechanism known as the “cochlear amplifier”. As a consequence of this active process, the sensitivity and the frequency discrimination of the hearing system are greatly increased (1). Damage of this mechanism, for instance by acoustic trauma, aminoglycoside antibiotics or simply aging, is a common cause of sensory-neural hearing loss afflicting millions of people around the world.

At the core of the cochlear amplifier are the outer hair cells (OHCs). OHCs are specific to the mammalian cochlea, probably reflecting an adaptation to the frequency and dynamic range demands of mammalian hearing (2). They are cylindrical, with lengths ranging between 10 to 100 µm and a rather constant diameter of approximately 8 µm. Cochlear OHCs can reversibly change their length by two different mechanisms: slow and fast OHC motility (3). Slow OHC motility occurs in seconds and involves cytoskeletal reorganization (4). In contrast, fast motility works in the microsecond range and is voltage-driven, with hyperpolarization causing elongation and depolarization shortening of the OHCs (5-7). We and others have recently demonstrated that OHC fast motility is mediated by the concerted direct action of a large number of independent molecular motors embedded in the OHC lateral plasma membrane (8-11), and funneled along the cell longitudinal axis by the prominent actin-spectrin cortical cytoskeleton (12).

Compelling evidence suggests that the gain of the cochlear amplifier is regulated in vivo through the modulation of OHC motility by acetylcholine (ACh) released from terminals of the

‡ Abbreviations: OHCs, outer hair cells; ACh, acetylcholine; OC, organ of Corti; GTPγS, guanosine 5'-O-(3-Thiotriphosphate); GDPβS, guanosine 5’-O-(2-Thiodiphosphate)
medial efferent system (for review, see (13,14)). Little is known, however, regarding the molecular mechanisms activated by ACh in OHCs. Several lines of evidence have led us to consider the involvement of members of the Rho (Ras-homologous) family of small GTPases in this process. For instance, early studies have established that RhoA, Rac1 and Cdc42 play a crucial role in cytoskeletal reorganization and mediate different types of motility in non-auditory cell populations (for review see (15,16)). In addition, recent evidence has indicated that ACh activates Rho-mediated signaling pathways in neuroblastoma cells (17). More importantly, targets of Rho GTPases have been associated to sensorineural hearing loss. For example, a mutation in the Dia1 protein (a profilin-ligand and target of Rho (18,19)) is the cause of the autosomal dominant nonsyndromic deafness DFNA1 (20), and mutations in another potential target of Rho, Myosin VIIa, are responsible for human Usher syndrome type 1B (21-23). The existence of a direct link between these pathways, OHC motility, and the physiology of the hearing system, however, remains unexplored. Thus, this is the first study to demonstrate that Rho proteins participate in the signaling cascade that ultimately regulates OHC motility in response to ACh. This finding is crucial for our understanding of a basic mechanism for both normal human hearing and deafness.
EXPERIMENTAL PROCEDURES

Immunofluorescence and western blot analyses. Subcellular localization of RhoA, Rac1, and Cdc42 was determined by confocal microscopy as previously described (24). Antibodies against RhoA were obtained from Santa Cruz (Santa Cruz, CA), and those against Rac1 and Cdc42 from Transduction Labs (Lexington, KY). Samples were double-labeled with the actin probe Rhodamine-phalloidin (Molecular Probes, Eugene, Oregon), and visualized using a Zeiss LSM-410 confocal microscope with objectives Plan-Apo 63X (NA=1.4) and C-Apo 40X (NA=1.2). For western blot analysis, total cell homogenates from cochlea and brain of guinea pigs were separated by SDS-PAGE (30µg protein/lane), transferred to nitrocellulose membranes and incubated with the individual antibodies described above. The reaction was detected by enhanced chemiluminescence using a peroxidase-labeled secondary antibody (Amersham, UK).

Measurement of OHC motility. OHCs were isolated from cochleas of young (200-300 g) guinea pigs by microdissection, and suspended in Leibowitz L-15 media (Gibco, Gaithersburg, MD) in a perfusion chamber on an Axiovert 135 inverted microscope stage. Only cells that met established criteria for healthy OHCs were used in these studies (25). L-15 media was continuously renovated at a rate of 50 µl/min using a two-way perfusion system (KDS-120, KD Scientific, Boston, MA). OHCs were patch-clamped (-70mV rest potential; 4-5 MΩ pipet access resistance) at or immediately above the nuclear region using an EPC-9 amplifier (HEKA, Germany) and a Patchman electronic micromanipulator (Eppendorf, Germany). Patch pipets were filled with internal perfusion buffer (KF, 120mM; KCl, 20 mM; MgCl₂, 2 mM; HEPES, 10 mM) adjusted with Trizma Base to pH 7.4 and with glucose to 300 mOsm. Cells were perfused through the same patch pipet with the following compounds (either alone or combined) dissolved in
perfusion buffer: 100 µM GDPβS, GTPγS, or exoenzyme C3 from *Clostridium botulinum* (specific RhoA inhibitor (26). Calbiochem, San Diego, CA), 10 ng/ml toxin B from *Clostridium difficile* (specific inhibitor of RhoA, Rac1 and Cdc42 (27). List Laboratories, CA), and 100 µg/ml dominant negative (dnRac1 and dnCdc42 (28)) or constitutively activated (RhoAQL, Rac1QL and Cdc42QL (29)) mutants of RhoA, Rac1 and Cdc42 constructed by site-directed mutagenesis of Ser to Asn at codon 17, or Gln to Leu at codon 63, respectively (constructs kindly provided by Dr. J. Silvio Gutkind, NIDCR-NIH). Mutant proteins were prepared according to the method described by Grieco and co-workers (30) using glutathione Sepharose 4B (Pharmacia Biotech). GTPγS [100 µM] was always co-perfused with the inhibitors and activators of Rho GTPases either to reconstitute the cellular response to ACh as well as to favor mutant proteins in their competition with the endogenous Rho GTPases. In a few experiments, commercially available dominant negative and constitutively activated mutants of the small GTPases (Cytoskeleton, Denver, CO) were used with similar results.

After being patch-clamped, cells were permitted to stabilize in their new mechanical conditions for 8 to 10 minutes. Subsequently, Leibowitz L-15 and ACh (100 µM. Sigma, St Louis, MO) were delivered to the basolateral wall of the cells (~0.15µl/sec) using a computer-controlled perfusion system (DAD-12, ALA Scientific Instr., Westbury, NY). Cells were first perfused with L-15 during two consecutive periods of 73 sec each to detect any response associated either with the experimental procedure or the stimulation of stretch-activated channels known to be present in the OHC lateral wall (31). During this initial phase OHCs were electrically stimulated four times with bursts of three depolarization (+50 mV)/hyperpolarization (-140 mV) cycles to elicit fast motility, a procedure that contributes to a faster mechanical stabilization of the cells. Next, cells were externally perfused for 98 seconds with either L-15 or
ACh, and electrically stimulated as described above. Results plotted in figs. 2 and 3 correspond to the changes in cell length (slow motility) or fast motility amplitude induced by L-15 (Control) or ACh during this period. Finally, another electrical stimulation was performed during an additional perfusion with L-15 in order to test cell recovery. The osmolarity of every solution used in these experiments was controlled and adjusted 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 using Adobe Photoshop 4.0 (Adobe Systems, Inc., San José, CA), and the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Changes in total cell length and fast motility amplitude were measured as described elsewhere (32) with a resolution better than 0.1 µm. Values were expressed as percentages of total cell length (slow motility) or fast motility amplitude measured in the same cells immediately before external perfusion with L-15 (Control) or ACh. Arcsine transformed data from a total of 350 cells was statistically analyzed with ANOVA techniques by using the software StatView 4.1 and SuperAnova (Abacus, Berkeley, CA).
RESULTS

*RhoA, Rac1 and Cdc42 are expressed in guinea pig OHCs.* The expression pattern of RhoA, Rac1 and Cdc42 in guinea pig organ of Corti (OC) was investigated using western blot analysis and confocal microscopy. Figure 1 shows that the immunoreactivity for these proteins was more intense in OHCs than in other OC cell populations. In addition, there is a clear co-localization of these small GTPases with the OHC’s actin cytoskeleton, especially at the cuticular plate, infracuticular network and basolateral wall.

Subsequently, we explored the regulatory role of RhoA, Rac1 and Cdc42 on OHC motility. Changes in cell length (slow motility) and in the amplitude of electrically induced movement (fast motility) were measured in isolated OHCs patch-clamped in the whole-cell mode, internally perfused with different pharmacological and molecular inhibitors and activators of that small GTPases, and externally stimulated with ACh as described in Methods.

**ACh and OHC Slow Motility:** The involvement of G proteins in the modulation of OHC motility by ACh was suggested by preliminary results showing a progressive loss of response to ACh in patch-clamped OHCs. Reversal of this trend by the addition of either GTP or GTPγS to the pipet buffer, indicated that a relative depletion of endogenous GTP due to diffusion into the patch pipet may be occurring during the experimental procedure, and that GTP was essential for the OHC response to ACh. In our experimental conditions, and in the presence of GTPγS in the patch pipet, ACh induced a significant shortening (equivalent to -0.6±0.1% of the total cell length) in OHCs. In contrast, ACh did not induce any change in OHC motility either in absence of GTPγS or its replacement by GDPβS (Fig. 2A). Even though these results confirm the involvement of GTP binding proteins in the response of OHC to ACh, they give no clues about
their identity. In a subsequent experiment, however, we found that Toxin B abolished the ACh-induced OHC shortening (Fig. 2A). This result implicates Rho-mediated signaling cascades in this phenomenon, and also demonstrates that if other G proteins that can be activated by GTPγS are involved in this signaling cascade, they should be upstream of the Rho GTPases.

Subsequently, we tested the participation of individual members of the Rho family of GTPases in the ACh-activated pathway by using the inhibitor molecules dnRac1 and dnCdc42, and the exoenzyme C3 from *Clostridium botulinum* (Fig. 2B). dnRac1 and dnCdc42—individually—abolished the ACh-induced cell shortening, suggesting that this response may be mediated by a signaling cascade involving both Rac1 and Cdc42. In contrast, inhibition of RhoA by C3 enhanced cell shortening (from -0.6±0.1% to -1.1±0.1%). Together these results demonstrate that RhoA, Rac1, and Cdc42 are crucial components of the molecular machinery that mediates ACh-induced slow motility, and suggest that elongation signals mediated by RhoA counterbalance the cell shortening induced by the activation of a Rac1/Cdc42-mediated signaling cascade.

To better define the functional interactions between these GTPases in mediating ACh-induced OHC motility, we perfused isolated cells with combinations of inhibitor molecules and measured changes in OHC length (Fig. 2C). The inhibition of RhoA- and Cdc42-mediated signals by C3 and dnCdc42 resulted in a marked ACh-dependent shortening (-2.2±0.4%) suggesting that Rac1 is a major mediator in this process. We also explored the contribution of Cdc42 to the ACh-induced OHC motility by inhibiting both RhoA and Rac1 using C3 and dnRac1. Under this experimental condition, we did not observe any change in the length of OHC in response to ACh. This data indicates that the participation of Cdc42 in ACh-induced OHC slow motility—detected using the dnCdc42 mutant alone—is not direct but, likely, mediated by Rac1.
Subsequently, the inhibition of Rac1 and Cdc42 by dnRac1 and dnCdc42 was used to define the contribution of RhoA to this phenomenon. Interestingly, we did not observe any response to ACh under this condition. Since our previous observations using C3 alone indicated that RhoA stimulation by ACh should induce OHC elongation (Fig. 2B), this lack of response to ACh suggests that RhoA may be working either upstream or downstream of Rac1. The described experiments do not permit to distinguish between these two possibilities.

Next, we investigated the response to ACh of OHCs internally perfused with the constitutively activated mutants RhoAQL, Rac1QL and Cdc42QL (Fig. 2D). We did not observe ACh-induced changes in OHC slow motility in any of these three experimental conditions. These results suggest that the effect of the constant activation of one of the small GTPases may be counterbalanced by the internal activation of one of both of the others. An overstimulation of the full system by ACh should be not enough to shift the response away from equilibrium.

Lastly, we measured the response of OHCs co-perfused with inhibitors and activators of RhoA, Rac1 and Cdc42 (Fig. 2E). We found that co-perfusion of dnCdc42 and RhoAQL induced a significant, ACh-dependent OHC elongation (1.3±0.1%), in contrast to the absence of response observed in cells perfused with dnCdc42 or RhoAQL alone. This result further support the existence of a RhoA-mediated signaling pathway that induces OHC elongation and suggests that it may be counterbalanced by a Cdc42-mediated shortening. The inhibition of this ACh-dependent, RhoAQL-mediated elongation by co-perfusion with dnRac1 and RhoAQL, in turn, supports the idea that RhoA-mediated signals may be under Rac1 control, with RhoA upstream of Rac1. Similarly, the shortening observed in cells co-perfused with dnCdc42 and Rac1QL (-0.9±0.1%) and the lack of response to ACh in cells co-perfused with dnRac1 and Cdc42QL confirms the major role of Rac1 in this ACh-induced cell response, and suggests that Cdc42 is
also working upstream of Rac1. Interestingly, the inhibition of the C3-induced shortening by either Rac1QL or Cdc42QL suggests the existence of a negative feedback mechanism aimed at preventing overstimulation of the signaling pathway (Fig. 2F). Importantly, a similar coordination of signals between RhoA, Rac1 and Cdc42 has been proposed to explain cytoskeletal reorganization in neuroblastoma, fibroblast and hematopoietic cells (17,33,34).

**ACh and OHC Fast Motility:** The effect of ACh on OHC fast motility was investigated using a similar experimental design to that described for slow motility. In this system, the internal perfusion of OHCs with dnRac1 resulted in an increase of 10±1% in the amplitude of fast motility (Fig. 3B). This result suggests that Rac1 controls an ACh-activated pathway aimed at increasing the amplitude of fast motility. The simultaneous inhibition of Rac1, RhoA and Cdc42 by Toxin B, on the other hand, abolished the increase in amplitude induced by the inhibition of Rac1 alone (Fig. 3A). In consequence, this increase in amplitude must be mediated by RhoA, Cdc42, or both. Therefore, we examined the role of RhoA and Cdc42 in fast motility using C3 and dnCdc42. Interestingly, no ACh-dependent effects on the amplitude of OHC fast motility were detected in cells perfused with either of these inhibitors (Fig. 3B). Thus, these results suggest that the ACh-induced contributions of RhoA or Cdc42 to OHC fast motility are under Rac1 control.

We next perfused OHCs with a combination of inhibitor proteins. As shown in Fig. 3C, ACh increases the amplitude of fast motility by 11±2% in cells co-perfused with dnRac1 and C3. This is similar to the response induced by dnRac1 alone. This result indicates that the increase in amplitude induced by ACh in cells perfused with dnRac1 is not mediated by RhoA but by Cdc42. This conclusion is further supported by the lack of response to ACh of OHCs co-perfused
with dnRac1 and dnCdc42. In addition, ACh does not induces any response in cells co-perfused with C3 and dnCdc42, suggesting that Rac1 does not affect directly OHC fast motility.

Subsequently, we investigated the response to ACh of OHCs internally perfused with constitutively activated mutants of Rho GTPases. As shown in Fig. 3 D, ACh significantly decreases fast motility amplitude in cells internally perfused with Cdc42QL (-14.6±0.4%). This response can be made compatible with previous results suggesting that Cdc42 mediates the increase in fast motility amplitude, assuming that ACh stimulation induces a RhoA- and Rac1-mediated response aimed at counterbalancing the effect of Cdc42QL. In support of this hypothesis, we have found that internal perfusion of OHCs with Cdc42QL increases fast motility amplitude —independently of ACh— from 3.4±0.2% (Control) to 4.4±0.3% of the total cell length (Zhang and Kalinec, in preparation). Therefore, ACh should be contributing to the regulation of the system returning fast motility amplitude back to normal levels. In contrast to its effect on Cdc42QL-perfused cells, ACh does not induces significant changes in fast motility amplitude either in RhoAQL- or Rac1QL-perfused cells (Fig. 3D), suggesting that neither Rac1 nor RhoA affect directly OHC fast motility.

Finally, we investigated the behavior of OHCs perfused with a combination of inhibitors and activators of RhoA, Rac1 and Cdc42. Co-perfusion of Cdc42QL and dnRac1 did not change the ACh-induced increase in the amplitude of fast motility observed in cells perfused with dnRac1 alone (9±2%. Compare Figs. 3B and 3E). This result may also be described as a dnRac1-mediated inhibition of the regulatory mechanism counterbalancing the effect of Cdc42QL (compare Figs. 3D and 3E). Both views are congruent with a model where Cdc42 mediates directly an ACh-induced increase in OHC fast motility, while Rac1 is the crucial component of the regulatory mechanism and controls the RhoA-mediated pathway. The proposed roles for
Cdc42 and RhoA increasing and decreasing OHC fast motility amplitude, respectively, are further supported by the findings that ACh induces a significant decrease in the amplitude of fast motility in OHCs co-perfused with dnCdc42 and RhoAQL (-10.0±0.3%. Fig. 3E) but lack of effect on cells co-perfused with dnRac1 and RhoAQL.

The crucial role of Rac1 is further emphasized by the observed effect of co-perfusing Rac1QL with either C3 or dnCdc42 (Fig. 3E). Co-perfusion of dnCdc42 and Rac1QL resulted in an ACh-dependent increase in fast motility amplitude (9±1%), whereas co-perfusion of C3 and Rac1QL induces a perceptible (even though not statistically significant) decrease in amplitude. These results suggest that inhibition of Cdc42 induces a Rac1-mediated downregulation of the pathway that activates RhoA and the simultaneous stimulation of the pathway that activates Cdc42. Conversely, inhibition of RhoA should be able to induce a Rac1-mediated downregulation of the pathway that activates Cdc42 and the simultaneous stimulation of the pathway that activates RhoA. This hypothesis is further supported by the lack of response to ACh observed in cells co-perfused with C3 and Cdc42QL. Therefore, Rac1 should be working as a master control of the ACh-activated pathway (Fig. 3E).
DISCUSSION

By using specific activators and inhibitors of RhoA, Rac1 and Cdc42, we have revealed the first evidence that modulation of OHC motility by ACh is mediated by Rho GTPases. Our results suggest that Rac1 is a crucial regulator of ACh-induced OHC motility. In cooperation with Cdc42, Rac1 mediates OHC shortening and a simultaneous increase in the amplitude of OHC. In contrast, in cooperation with RhoA, Rac1 mediates OHC elongation and a decrease in the amplitude of fast motility. Furthermore, these results indicate the existence of a Rac1-controlled feedback mechanism responsible for the fine-tuning of OHC fast motility and able to rapidly revert the changes induced by ACh. These processes are essential for maintaining the homeostasis of the cochlear amplifier and thereby for normal hearing.

Localization of RhoA, Rac1 and Cdc42 in OHCs

In OHCs, RhoA, Rac1 and Cdc42 co-localize with cytoskeletal structures. Immunolabeling was stronger at the cuticular plate, infracuticular network, and along the OHC’s lateral wall (Fig. 1). The cuticular plate is a dense meshwork of actin and spectrin in the OHC apex, thought to be a stiff non-flexible plate in which the stereocilia are anchored (35). The infracuticular network, in turn, is an expansion of the cuticular plate into the cytoplasm found only in OHCs from the apical end of the guinea pig cochlea (such as those illustrated in Fig. 1) (36). The OHC lateral wall, on the other hand, is a unique structure composed of three distinct layers: the plasma membrane, the cortical cytoskeleton and the lateral cisternae (3). The lateral cisternae are multiple, highly ordered layers (as many as twelve in guinea pig) lining up the lateral cytoplasmic surface of OHCs from the apical tight junction to the infranuclear region (37,38). While in OHCs the Golgi apparatus is small and confined to a restricted region in the apical, sub-
cuticular area of the cell, specific labeling suggests that lateral cisternae membranes share characteristics of Golgi and smooth endoplasmic reticulum (39,40). This is particularly relevant, since localization analyses in other laboratories have shown association of Cdc42 with Golgi membranes (41,42).

The cortical cytoskeleton, located in the narrow space (~30nm width) between the plasma membrane and the outermost cisternal membrane, is a two-dimensional structure responsible for the shape and most of the mechanical properties of the OHCs (3,12). It is composed essentially by roughly circumferential actin filaments up to 1µm long, cross-linked by shorter (~50nm) spectrin tetramers (12). The actin filaments are connected to the plasma membrane through thousands of 25 nm long, rod-like structures (pillars) placed about 40 nm from each other (12,43,44). Even though no changes in the distribution of Rho GTPases or actin were detected in the lateral wall of OHC after stimulation with ACh, Rho-mediated changes in the cortical cytoskeleton remains one of the most attractive candidate mechanisms for the regulation of OHC motility. For instance, subtle biochemical changes in the OHC cortical cytoskeleton may be undetectable by the techniques used in the present work. Future detailed biochemical analyses of this phenomena will likely provide critical insights into the identity of the molecular targets of the Rho GTPases.

**ACh, Rho GTPases and OHC motility**

OHC slow motility is an actin-mediated process. Thus, the putative involvement of RhoA, Rac1 and Cdc42 in its regulation should hardly be a surprise. OHC fast motility, on the other hand, is independent of ATP, Ca²⁺ and, presumptively, of any second-messenger mediated
process (3,7,8). The motor function is very robust, and neither drugs like cytochalasins, colchicine and nocodazole, nor complete disruption of cytoplasmic structures by internal perfusion of the cells with high concentrations of trypsin, can inhibit it (3,8,10,11). However, regulation does not imply inhibition, and OHC fast motility could be regulated without inhibition of the motor function. The mechanical load on the membrane-embedded motor proteins, for instance, could be modulated through changes in number and strength of the thousands of periodically distributed “pillars” that connect the cortical cytoskeleton to the plasma membrane in OHCs. Interestingly, the membrane-cytoskeleton linkage through members of the FERM (Four-point-one/Ezrin/Radixin/Moesin) protein family seems to be regulated by Rho GTPases (16,45,46), and FERM proteins have been associated with the pillars in guinea pig OHCs (47).

In non-auditory cell types Rho GTPases have been associated with a variety of motile processes such as filopodia, lamellipodia, and stress fiber formation (15,16). Mature OHCs, however, are terminally differentiated, highly specialized cells that do not migrate, divide, or form these structures. Therefore, it is likely that in OHCs, Rho proteins have adapted to regulate functions that are unique to these cells. The recent report that mutations in known targets of Rho GTPases result in deafness further substantiates this idea. In this regard, our results demonstrate that Rho GTPases are involved in the regulation of OHC motility by ACh, a crucial mechanism for acoustic signal amplification and frequency discrimination in the mammalian inner ear. The role of RhoA, Rac1 and Cdc42 in this process, however, may be much more complex than the work models depicted in figs. 2F and 3F suggest. For instance, dominant-negative mutants of Rho GTPases inhibit the catalytic domain of Rho-GEFs rather than Rho themselves (28). Since it
has been demonstrated that some of these GEFs may activate more than one Rho-family member (48,49), dominant-negative mutants might be interfering with the activation of other components of the family in addition to its normal counterpart, generating a more complex scenario for GTPase interplay. Future studies focused on identifying and characterizing the Rho targets in OHCs will undoubtedly help to refine these models as well as to provide critical insights into the basic mechanisms of both normal human hearing and deafness.
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Fig. 1: **Double-labeling of Rho GTPases and the actin cytoskeleton in guinea pig’s organ of Corti and isolated OHCs.** Optical sections of whole mount preparations of organ of Corti (showing the characteristic three parallel rows of OHCs), isolated OHCs and western blot analysis, confirm the expression of these GTPases in guinea pig cochlea. Top panels correspond to samples labeled with the antibodies against RhoA (A), Rac1 (B) and Cdc42 (C), visualized with FITC-conjugated secondary antibodies (green fluorescence). Middle panels correspond to the same samples but showing the actin cytoskeleton labeled with the actin probe rhodamine phalloidin (red fluorescence). The bottom panels depict both images superimposed, showing in yellow the regions of co-localization of Rho GTPases and the actin cytoskeleton.

Fig. 2: **Effect of ACh on OHC slow motility.** (A-E): Patch-clamped cells were externally perfused with Leibowitz L-15 (Control) or ACh, while internally perfused through the patch pipet with inhibitors and activators of Rho GTPases. Changes in cell length (slow motility) were measured at rest potential, and expressed as percentages respect to the total cell length at rest potential immediately before external perfusion of the cells with either Leibowitz L-15 (Control) or ACh. Error bars represent standard error of the mean (SEM). * = P<0.05. (F): one possible model of the ACh-activated, Rho-mediated pathway regulating OHC slow motility. ACh activates a Cdc42-mediated pathway that induces OHC shortening, and a RhoA-mediated pathway that generates signals aimed at elongating the OHC. Rac1, working downstream both RhoA and Cdc42, controls the cell mechanical response. Cell elongation or shortening will depend on the balance between the Cdc42-mediated (shortening) and the RhoA-mediated
(elongation) signals. In addition, we propose the existence of a negative feedback mechanism mediated by Cdc42, aimed at preventing excessive cell shortening or elongation. Question marks indicate other (still unknown) molecular components of the ACh-activated signaling cascade that modulates OHC slow motility.

Fig. 3: **Effect of ACh on OHC fast motility.** (A-E): Patch-clamped cells were externally perfused with Leibowitz L-15 (Control) or ACh, while internally perfused through the patch pipet with inhibitors and activators of Rho GTPases. Changes in fast motility amplitude for each cell are expressed as percentages respect to the amplitude measured in the same cell immediately before the external perfusion with either Leibowitz L-15 (Control) or ACh. Error bars represent standard error of the mean (SEM). * = P<0.05. (F): one possible model of the ACh-activated, Rho-mediated pathway regulating OHC fast motility. ACh activates a Cdc42-mediated pathway that induces an increase in OHC fast motility amplitude. In addition, a RhoA-mediated pathway —with RhoA upstream Rac1— generates signals aimed at decreasing the amplitude of OHC fast motility. Rac1 should be the master control, regulating the changes in fast motility amplitude through the balance of the Cdc42-mediated (increase) and the RhoA-mediated (decrease) signals. Question marks indicate other (still unknown) molecular components of the ACh-activated signaling cascade that modulates OHC fast motility.
Fig. 2

(A) Graph showing the change in cell length (%) for different treatments: Control and ACh.

(B) Graph showing the change in cell length (%) for C3, dnRac1, and dnCdc42 with and without ACh.

(C) Graph showing the change in cell length (%) for C3, C3+dnRac1, and C3+dnCdc42 with and without ACh.

(D) Graph showing the change in cell length (%) for RhoAQL, Rac1QL, and Cdc42QL with and without ACh.

(E) Graph showing the change in cell length (%) for C3, C3+Rac1QL, C3+Cdc42QL, and C3+RhoAQL with and without ACh.

(F) Diagram illustrating the relationship between ACh-receptor, Plasma Membrane, Elongation, Shortening, RhoA, Rac1, and Cdc42, with feedback loops indicated.
Fig. 3
Rho GTPases mediate the regulation of cochlear outer hair cell motility by acetylcholine
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