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FINAL REPORT ON CONTRACT N00014-88-K-0078

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CONTRACTOR: Massachusetts General Hospital

CONTRACT TITLE: Adaptation and Regulation of the Transduction Mechanism in Vestibular Hair Cells

START DATE: 15 December 1987

END DATE: 14 December 1990

RESEARCH OBJECTIVES AND PROGRESS:

Aim 1

Quantification of Adaptation, and the Relaxation/Tensioning Hypothesis

a. To measure the timecourse of adaptation for hair bundle displacement steps of varying amplitude and duration, and to determine whether adaptation is complete or partial.

b. To measure the timecourse and extent of adaptation after initial displacement steps, in order to determine whether the adaptation to a subsequent step depends on initial hair bundle position.

c. To measure both adaptation rate and the resting displacement-response curve under conditions that alter adaptation, in order to understand whether the positive and negative adaptation rates uniquely determine the resting position of the displacement-response curve, and thus to understand the extent to which the relaxation/tensioning hypothesis explains the adaptation process.

Progress

The project went extremely well, and has developed into the most exciting work in our lab over the past few years. The aims evolved slightly as the project was carried out, but for the most part, the work progressed as proposed. In general, the relaxation/tensioning hypothesis has been confirmed:

a. The timecourse was measured for a variety of steps, and was characterized as an initial rate of adaptation vs. displacement. When plotted this way, the behavior was quite different for the positive and negative displacements of the hair bundle: positive steps elicited an adaptation dependent on the size of the step, whereas negative displacements elicited an adaptation whose rate was independent of the stimulus. These were interpreted in terms of the tip-links hypothesis, as a tension-dependent slipping rate of a motor molecule and as an intrinsic climbing rate of a motor. In other experiments, it was clear that adaptation is not complete, but is more complete for small displacements than for large, suggesting a limited spatial range of adaptive movements.

b. Specific experiments on adaptation after steps were not carried out, because it was apparent from the limited spatial range that rates must be different.

c. It was found that calcium concentration affected the rates, but affected slipping more than climbing, thereby offering a test of the hypothesis. In high calcium, slipping is faster, tension is expected to be less, and it was observed that channels are open less frequently. This all is consistent with the hypothesis. Initial qualitative confirmation was published, and more recent, quantitative tests also fit but are still in preparation.

These results were published in Hacohen, Assad, Smith & Corey (1989).

Aim 2

The Location and Mechanism of Calcium's Effect on Adaptation

a. To identify the site of action of calcium as either extracellular or intracellular, and then to localize the site in relation to structures associated with the sensory bundle.

b. To identify the biochemical nature of calcium's site of action, by studying the dose-response relation and the cationic specificity of the effect, and by studying the effect of inhibitors of calcium-binding proteins.

Progress

a. Experiments with single cells provided a very satisfying answer to the localization of calcium action. The effect of external calcium was only observed when the membrane potential was negative; when positive (so that the positively charged calcium would not enter the cell), the adaptation behaved as if calcium was very low. This (and other controls) indicates that calcium must enter the cell to reach its site. The path of entry is almost certainly the transduction channels at the tips of stereocilia.

The voltage dependence also enabled us to determine how far calcium must go to reach its site. The effect of calcium was observed within a few milliseconds after switching to a permissive voltage; in that time calcium could only diffuse 1-2 micrometers. Thus the site is inside the tips of the stereocilia.

These results were published in Assad, Hacohen & Corey (1989).

b. An intracellular site suggests a specific calcium-binding protein, rather than an electrostatic effect. We tried a number of different divalent cations, and found that none would replace calcium in potentiating adaptation, and several competitively inhibited. This is consistent with a specific binding site. We also tried inhibitors of calmodulin. Some calmodulin inhibitors did inhibit the adaptation, but results were inconsistent.

The results on divalent cations were published in Hacohen, Assad, Smith & Corey (1989).

Aim 3

Identification and Localization of Calcium-Binding Proteins and Structural Proteins that may be involved in Adaptation.

a. To determine the distribution of calmodulin-like immunoreactivity within the hair cell soma and within the hair bundle; similarly, to look for immunoreactivity to certain other calcium-binding proteins.

b. To determine by immunohistochemical methods whether the 110 kD protein of intestinal brush border is also in hair cell stereocilia; if not, to use antibodies to other calcium-dependent actin-binding proteins in order to identify the actin-to-membrane linkers seen with electron microscopy.

Progress

A remarkable trick for purification of pure stereocilia enabled us to get detailed information on some structural and calcium-binding proteins in the bundle. A graduate student worked out a method to stick all the stereocilia in a vestibular organ to nitrocellulose paper, and then to peel them off with the paper. The material could then be run out on gels for biochemical analysis. We then could do immunoblots on the gels, or immunocytochemistry on the cells.

a. Two calcium-binding proteins appeared in gels: calmodulin and calbindin. Calbindin occurs in very high abundance, and is distributed throughout the stereocilia. Calmodulin is much less abundant, but is found especially at the *tips* of the stereocilia, where the calcium-regulated adaptation mechanism is thought to be.

b. A large number of antibodies to the 110 kD protein, and to other myosins, were tried. None of the 110-kD antibodies bound, and the gels had no band at 110 kD. Anti-myosin antibodies did not bind, for the most part, but we found two that did. This is presently being pursued.

A related set of experiments asked whether myosin could actually move along the actin cores of the stereocilia, as one test of whether it could be the motor. Glass beads coated with chicken

muscle myosin did actually move along stereocilia that had been demembranated, and they moved at just the same rate as the climbing rate for adaptation. While not proof of a myosin involvement, the correspondence is very intriguing.

These results were published in Shepherd, Barres & Corey (1989), and in Shepherd, Corey & Block (1990).

INVENTIONS: While we have modified some standard techniques in novel ways, none would be considered patentable inventions.

PUBLICATIONS AND REPORTS:

Papers

Hacohen, N., Assad, J.A., Smith, W.J., and Corey, D.P. (1989) Regulation of tension on hair-cell transduction channels: Displacement and calcium dependence. J. Neurosci. 9: 3988-3997.

Assad, J.A., Hacohen, N., and Corey, D.P. (1989) Voltage dependence of adaptation and active bundle movement in bullfrog saccular hair cells. Proc. Natl. Acad. Sci. USA. 86: 2918-2922.

Shepherd, G.M.G., Barres, B.A., and Corey, D.P. (1989) "Bundle Blot" purification and initial protein characterization of hair-cell stereocilia. Proc. Natl. Acad. Sci. USA 86: 4973-4977.

Shepherd, G.M.G., Corey, D.P., and Block, S.M. (1990) Actin cores of hair-cell stereocilia support myosin motility. Proc. Natl. Acad. Sci. USA 87:8627-8631.

Abstracts

Shepherd, G.M.G., Barres, B.A., and Corey, D.P. (1988) "Bundle blot" purification of hair cell stereocilia: electrophoretic protein mapping. Soc. Neurosci. Abstr. 14:799. ((321.8)).

Assad, J.A., Hacohen, N., and Corey D.P. (1989) Localization and biophysical characterization of an adaptation mechanism in amphibian hair cells. J. Acoust. Soc. Am. Suppl. 1 85:L3.

Shepherd, G.M.G., Barres, B.A., and Corey, D.P. (1989) Mechanical purification and protein constituents of amphibian hair cell stereocilia. J. Acoust. Soc. Am. Suppl. 1 85:L8.

Block, S.M., Goldstein, L.S.B., Schnapp, B.J., Shepherd, G.M.G., Corey, D.P., Blair, D.F. and Berg H.C. (1990) Using optical tweezers to investigate mechanoenzymes in vitro.

Corey, D.P., Assad, J.A., Huang, P.L., Shepherd, G.M.G., and Block, S.M. (1990) An active motor mediates hair-cell adaptation, and could be myosin-like. Assoc. Res. Otolaryngol. 259.

TRAINING ACTIVITIES: John Assad, a graduate student in the Neurobiology Program at Harvard Medical School, worked on the physiology of this project and it formed the core of his PhD thesis. Gordon Shepherd and Philip Huang, both MD/PhD students at Harvard Medical School, worked on biochemical aspects of the project.

Women or minorities - 1
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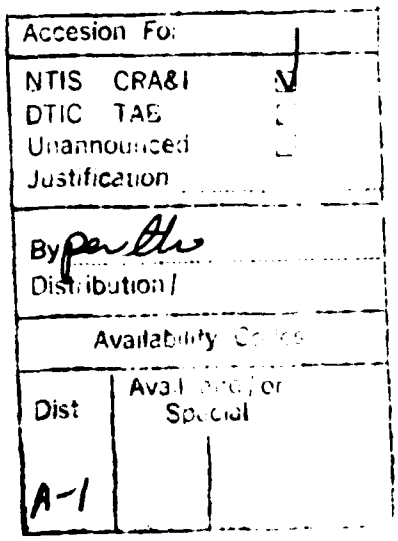

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Regulation of Tension on Hair-Cell Transduction Channels: Displacement and Calcium Dependence

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An epithelial preparation of the bullfrog sacculus was used to characterize the initial rate of the adaptation mechanism in hair cells and its dependence on displacement and calcium. The $I(X)$ curve relating transduction current and bundle displacement shifted along the X-axis without substantial change in slope, as previously observed, suggesting that adaptation involves a change in the attachment point of the elastic element connected to ion channels. If the "tip links" model of transduction is correct, this implies that one end of the link moves along the side of the stereocilium. The rates were highly asymmetric: in the tensioning direction the rate was roughly constant at 1–2 μm/sec (calculated as motion along a stereocilium); this is similar to that of myosin on actin. In the relaxing direction it appeared linearly dependent on tension. Calcium preferentially potentiated the relaxation, and apparently reduced the resting tension in the elastic element. The calcium site appears specific for calcium, as other divalent cations inhibited its action. Dihydrostreptomycin inhibited the positive rate, but its effect could not be explained by a simple channel block, and it seems inconsistent with screening of negative charge in the mouth of the transduction channel.

Tension exerted through some elastic linkage appears to be the essential stimulus for gating of transduction channels in vertebrate hair cells (Corey and Hudspeth, 1983b; Howard and Hudspeth, 1987). An attractive model supposes that fine filaments extending vertically from the tip of each stereocilium to the side of its taller, adjacent neighbor (the "strings" or "tip links") constitute the linkage to channels in the tips of stereocilia, and that lateral displacements of the stereocilia cause a relative shear at their tips to alter tension in the tip links (Hudspeth, 1982; Pickles et al., 1984). Because the transduction current is not zero at rest but is 20–40% of the maximum (Hudspeth and Corey, 1977), it is thought that some mechanism exerts a resting tension on transduction channels.

Following displacements that increase the current through transduction channels, the current declines towards the resting level over about 100 msec (Corey and Hudspeth, 1983a). The decline can be described operationally as a simple shift, along the displacement axis, of the curve relating displacement and transduction current [the $I(X)$ curve]; in this model the shift would correspond to a relaxation of tension in the linkage (Eatock et al., 1987). Similarly, a displacement that reduces transduction current is followed by an increase in current towards the resting level, corresponding to a restoration of linkage tension. Howard and Hudspeth (1987) observed changes in the stiffness of stereociliary bundles with a time course that matched that of the change in transduction current, further supporting the model. There appears to be a mechanism, then, that acts to keep constant the tension in the linkages attached to the ion channels. What is the molecular mechanism of the tension regulation system?

The mechanism is known to be sensitive to calcium concentration in the medium around the stereocilia (Eatock et al., 1987). Because the calcium sensitivity disappears at positive membrane potentials, the calcium site—and presumably the mechanism itself—is thought to be intracellular (Assad et al., 1989). Because the voltage dependence occurs almost instantly, the site is thought to be close to the transduction channels (within 1–2 μm) (Assad et al., 1989). Lastly, depolarization that increase the tension on ion channels, inferred physiologically, also cause motions of the hair bundles (Assad et al., 1989), suggesting that the tension-regulating mechanism is an active process that involves an energy-consuming motile element.

In this paper we report the displacement and calcium dependence of the mechanism. By measuring the initial rate of the mechanism we can infer some of its biophysical properties. These properties suggest that the mechanism is primarily sensitive to tension but is modulated by intracellular calcium. The results are consistent with a model (Howard and Hudspeth, 1987) that involves a change of the attachment point of the tip link, by a myosin-like molecule moving on the actin core of the stereocilium. A preliminary account of some of this work has appeared (Corey et al., 1987).

Materials and Methods

Microphonic preparation. We used the *in vitro* microphonic preparation of the bullfrog sacculus, essentially as described by Corey and Hudspeth (1983a, b) and by Eatock et al. (1987). Briefly, sacculi were dissected from bullfrogs (*Rana catesbeiana*) into a saline solution; the maculae were trimmed of surrounding tissue and mounted across a hole separating an apical and a basal chamber. Most of the otolithic membrane was peeled off hair bundles, leaving a patch of several hundred hair cells of similar orientation still attached in the periphery of the macula. Apical

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Neurobiology

Voltage dependence of adaptation and active bundle movement in bullfrog saccular hair cells

(mechanoreceptor/vestibular system/ion channel/cell motility)

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Communicated by Charles F. Stevens, January 3, 1989

ABSTRACT **Hair cells of the bullfrog sacculus adapt to maintained displacement stimuli in a manner that suggests an active regulation of the tension stimulus reaching transduction channels. We have examined adaptation in dissociated hair cells by whole-cell patch-clamp recording and video microscopy. Adaptation was present in these cells, and it depended on extracellular calcium. The adaptation rate—as well as the position of the resting current–displacement curve—also depended on membrane potential, suggesting that calcium passes into the cytoplasm to reach its site of action. After abrupt hyperpolarization, the adaptation rate increased within milliseconds, suggesting that the calcium site is within a few micrometers of the ion channels through which calcium enters. The voltage dependence of the resting current–displacement curve, together with the "gating springs" hypothesis for transduction, predicts movement of the bundle away from the kinocilium when the cell is depolarized. This was observed.**

The bullfrog sacculus is exquisitely sensitive to linear acceleration: vibratory accelerations of only 1 $mm \cdot s^{-2}$ saturate the output of the organ (1). Yet the static acceleration of gravity is 4 orders of magnitude greater. In fact, the receptor cells of the sacculus possess an extraordinary feedback mechanism—adaptation—which shifts the sensitive range of the cells within tens of milliseconds so as to cancel static biases. Adaptation has been studied most extensively by *in vitro* microphonic recording from the bullfrog sacculus (2, 3). In these experiments, which measure the receptor current of several hundred hair cells stimulated *en masse*, a maintained displacement of the stereociliary bundles elicits a transduction current, which declines to a steady level in ≈100 ms. This decrement in current is not an inactivation process; rather, it is due to a shift in the relation between bundle displacement and receptor current—the $I(X)$ curve—in the direction of the adapting step. If bundle displacement directly communicates tension to the transduction channels (4), then adaptation in response to positive displacements (toward the kinocilium) must act to relax the mechanical input, favoring reclosure of the channels, while adaptation to negative stimuli (away from the kinocilium) restores tension, favoring reopening. In fact, positive displacements produce a mechanical relaxation, which can be directly observed as an increase in bundle compliance with the same time course as adaptation (5).

Little is known of the cellular mechanism of adaptation. Higher extracellular calcium increases the rate of adaptation to both positive and negative stimuli and shifts the resting $I(X)$ curve so there are fewer channels open at rest (3, 6). In this study, we have examined the effects of membrane potential and extracellular calcium concentration on adaptation in single isolated hair cells from the bullfrog sacculus to localize the specific site of action of calcium. The results are consistent with a calcium site very near the transduction channels. They also predict a voltage-dependent bundle movement, which was observed with video microscopy. A preliminary report of this work has appeared (7).

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MATERIALS AND METHODS

Preparation. Saccular hair cells from adult bullfrogs (*Rana catesbeiana*) were dissociated following a procedure modified from Lewis and Hudspeth (8). Sacculi were exposed, and an oxygenated papain solution [0.5 mg of papain per ml (Calbiochem) and 2.5 mM L-cysteine] was dripped into the perilymphatic space for 30 min at room temperature; this procedure and all subsequent steps of the dissociation were done in a low-calcium saline containing 120 mM Na, 2 mM K, 0.1 mM Ca, 122 mM Cl, 3 mM dextrose, and 5 mM Hepes (pH 7.25). After a 1-min wash with bovine serum albumin (0.5 mg/ml) (Sigma), maculae were dissected from the frog into a dish and incubated for 30 min in bacterial protease type XXIV (50 μg/ml) (Sigma) to loosen the overlying otolithic membranes. Single cells were dissociated from the maculae with an eyelash and settled onto the clean glass bottom of the recording chamber. This procedure, in which papain was largely prevented from reaching the mechanically sensitive hair bundles by tight junctions between hair cells and supporting cells, resulted in a substantially higher proportion of transducing cells and larger transduction currents than with other protocols (8, 9).

Mechanical Stimulation. Mechanical displacements were delivered to the stereociliary bundle using a stiff glass probe driven by a two-dimensional piezoelectric bimorph stimulator that moved in the horizontal plane (10). The probe tip adhered directly to the kinociliary bulb; a photodiode provided an optical monitor of probe movement near its attachment to the bundle. In addition, the stimulus probe and attached stereociliary bundle were directly observed at high magnification (×10,000) with an analog-enhanced video camera system (Hamamatsu C2400) to assess the relative motion of the bundle and cell body.

Electrical Recording. Membrane currents were recorded by whole-cell patch-clamp recording (11, 12) with micropipettes attached to the basolateral membrane of the cell near the cuticular plate. Seals on this surface proved extremely stable: initial seal resistances were typically 20–50 GΩ, and it was not uncommon to record from a cell for >30 min. Currents were recorded with a Yale Mk.V patch-clamp amplifier equipped with a 1-GΩ head stage. When necessary, leakage, capacitive, and voltage-dependent currents were subtracted by presenting voltage steps with and without mechanical stimulation. Leakage currents at −80 mV were typically −100 pA. Residual series resistance after compensation was usually 6–8 MΩ. The current signal and photodiode signal were filtered at 5 kHz and digitized on-line with a PDP-11/73 computer system (Indec Systems, Sunnyvale, CA); the

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Cell Biology

"Bundle blot" purification and initial protein characterization of hair cell stereocilia

(mechanotransduction/auditory system/vestibular system/cytoskeleton/immunocytochemistry)

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Communicated by Thomas S. Reese, April 4, 1989

ABSTRACT **Stereocilia were isolated from bullfrog (*Rana catesbeiana*) saccular hair cells by nitrocellulose adhesion. The high purity and high yield of the preparation were demonstrated by microscopy. SDS/PAGE of stereociliary proteins resolved 12–15 major bands. Actin, previously identified as a component of the stereociliary core, was identified in purified stereocilia as a band comigrating with authentic actin and by phalloidin labeling of intact isolated stereocilia. Fimbrin was identified in immunoblots of purified stereocilia. The most abundant other proteins migrated at 11, 14, 16–19, 27, and 36 kDa. Demembranated stereociliary cores consisted primarily of protein bands corresponding to actin and fimbrin and several proteins ranging from 43 to 63 kDa. Because the adaptation mechanism in hair cells is calcium-sensitive and seems localized to stereocilia, we sought evidence for calcium-binding proteins in stereocilia. Calmodulin and calbindin antibodies labeled stereocilia in intact cells. A protein band in purified stereocilia exhibited a Ca^{2+}-dependent shift in electrophoretic mobility identical to that of authentic calmodulin, and the 27-kDa band may represent calbindin. These biochemical data demonstrate that stereocilia consist of a relatively small set of proteins. Most of these, including those involved in transduction and adaptation, are as yet uncharacterized. The availability of purified stereocilia should prove useful in further studies of structure–function relationships in these mechanically sensitive organelles.**

Hair cells, the receptor cells of the auditory and vestibular systems, transduce displacements of their apical hair bundle into electrical signals. The stereocilia constituting the bundle, which share structural properties with intestinal microvilli and growth-cone filopodia, are the mechanically sensitive organelles: displacement is thought to alter tension on filamentous links between their tips, which directly opens ion channels (1–3). The transduction current adapts to maintained stimuli, through a tension-altering mechanism (4, 5) thought to be situated in the tips of stereocilia (6).

Although the physiology of transduction and adaptation is understood in some detail, the proteins involved in these processes are largely unknown. Because of the difficulty in purifying sufficient amounts of stereocilia, protein identification has been pursued through immunocytochemistry. Thus the structural proteins actin (7, 8) and fimbrin (8) have been detected. Calbindin-28 immunoreactivity has been detected in rat and cat stereocilia (10, 11). Some physiological evidence points to the presence of calmodulin (CaM) in stereocilia: the adaptation process in bullfrog vestibular cells is sensitive to Ca^{2+} (4) and is blocked by CaM antagonists (12). Yet CaM antagonists are not particularly specific and could inhibit other calcium-binding proteins.

To study the biochemistry of stereocilia we developed a purification technique that exploits the adhesion of the apical ends of stereocilia to nitrocellulose paper and the mechanical fragility of the narrow basal ends. A related strategy has been used to adsorb small numbers of piscine stereocilia onto coverslips for ultrastructural studies (13). The nitrocellulose adhesion method, which we term "bundle blot" purification, gave a sufficiently high yield of pure stereocilia for biochemical analysis. Some results have appeared in preliminary form (14).

METHODS

Blotting Stereocilia onto Nitrocellulose. Bullfrogs (*Rana catesbeiana*) were purchased from Ming's Market (Boston, MA) and other suppliers and kept at 20°C. Frogs were pithed and decapitated, and the sacculi were rapidly removed and transferred to a cold (4°C) physiological buffer containing 50 μM Ca^{2+} and protease inhibitors (0.15 μM aprotinin, 20 μM leupeptin, 0.15 μM pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride); this cold buffer was used throughout the dissection. The saccular maculae were trimmed of surrounding epithelium and otolithic membranes were carefully removed with forceps. The apical surfaces were briefly washed with a gentle stream of dissection buffer. To harvest stereocilia, the apical surfaces of the maculae were blotted onto 1-mm^2 pieces of nitrocellulose paper (Schleicher & Schuell; 0.45-μm pores) and removed. Initially a piezoelectric bimorph configured as a force transducer was used to determine the optimal blotting force; thereafter this force of 5–20 mN (equivalent to 0.5–2 g) could, with practice, be delivered manually. The nitrocellulose papers with the adherent bundles were immediately (<60 s) processed for microscopy or biochemistry. Macular epithelia (hair-cell and supporting-cell somata, but not nerve) and otolithic membranes were collected onto similar small squares of nitrocellulose paper.

Characterization of Purity and Yield. Phalloidin, a toadstool alkaloid that binds F-actin, was used to monitor blotting efficiency. Samples were incubated in a solution containing formaldehyde (3.7%), lysolecithin (0.01%), and rhodamine-phalloidin (50 units/ml; Molecular Probes) for 30 min at room temperature and examined with fluorescence microscopy. For scanning electron microscopy, samples were fixed in 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide, dehydrated in ethanol, critical-point-dried, and coated with gold/palladium. An AMR-1000 scanning electron microscope was operated at 20–30 kV.

Demembranation. A membrane extraction method adapted from refs. 15 and 16 was used to isolate stereociliary cores. After stereocilia were blotted onto nitrocellulose paper, the paper was transferred directly into extraction buffer (1% Triton X-100/100 mM NaCl/1 mM $MgCl_2$/0.1 mM EGTA/10 mM Tris, pH 7.6). The detergent-insoluble cores, still attached to the paper, were first washed in "core buffer" (75 mM

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Abbreviation: CaM, calmodulin.

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Cell Biology

Actin cores of hair-cell stereocilia support myosin motility

(adaptation/cytoskeleton/optical tweezers/auditory system/vestibular system)

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Communicated by Howard C. Berg, July 18, 1990 (received for review May 29, 1990)

ABSTRACT **The actin cores of hair-cell stereocilia were tested as a substrate for the movement of myosin-coated beads in an *in vitro* assay. Large numbers of stereocilia from bullfrog sacculi and semicircular canals were isolated by blotting onto coverglasses and were demembranated to expose the polar actin tracks of their cytoskeletal cores. Silica or polystyrene beads, coated with thick filaments of chicken skeletal muscle myosin, were added to this core preparation in the presence of ATP. Myosin-coated beads could reach some of the cores by diffusion alone, but the efficiency and precision of the assay were improved considerably by the use of "optical tweezers" (a gradient-force optical trap) to deposit the beads directly on the cores. Beads applied in this fashion bound and moved unidirectionally at 1–2 μm/s, escaping the retarding force of the trap. Actin filaments within the stereocilia are cross-linked by fimbrin, but this did not appear to interfere with the motility of myosin. Beads coated with optic-lobe kinesin were also tested for movement; these bound and moved unidirectionally at 0.1–0.2 μm/s when applied to microtubule-based kinociliary cores, but not when applied to actin-based stereociliary cores. Our results are consistent with, and lend support to, a model for hair cell adaptation in which a molecular motor such as myosin maintains tension on the mechanically gated transduction channels. Optical tweezers and video-enhanced differential interference contrast optics provide high efficiency and improved optical resolution for the *in vitro* analysis of myosin motility.**

Hair cells, the receptor cells of the vertebrate inner ear, transduce mechanical displacements of their hair bundles into electrical signals. The hair bundle of each cell consists of 30–300 actin-based extensions, called stereocilia, and a single microtubule-based kinocilium. The one or two transduction channels at the tip of each stereocilium (1, 2) are gated by displacements of the bundle (3, 4). Tension stimuli are thought to reach the channels via fine filaments extending from the tip of each stereocilium upward to the side of its taller, neighboring stereocilium (reviewed in ref. 5). Ultrastructurally, these filaments have been described as "tip linkages" (6); physiologically, they behave as elastic "gating springs" (7).

The resting tension of the gating springs, and hence the channel open probability, is regulated by an adaptation process (8–10). A maintained displacement of the hair bundle toward its taller side elicits a depolarizing current that declines, over tens of milliseconds, toward the resting level. Similarly, displacement in the opposite direction elicits a rapid hyperpolarizing reduction in the transduction current, followed by a slower recovery to the resting level (9). This recovery suggests that the gating springs are actively retensioned. Assad *et al.* (11) found that voltage changes that alter calcium entry through transduction channels induce active bundle movements. These movements have a magnitude, direction, and time course similar to adaptation, suggesting that the two phenomena are generated by the same active process (11, 12). It has been proposed that a myosin-like molecule, attaching the tip linkage to the side of the taller stereocilium and moving along the actin core, could serve as the putative adaptation motor. Such a motor, attempting to move toward the tip of the stereocilium, would maintain tension in the tip linkage (7, 10).

The mechanoenzymatic properties of different myosins are intrinsic and essentially independent of the type of actin filament on which they move in *in vitro* experiments (13); actins, which are highly conserved, are equally effective substrates for all myosins. Within a cell, however, different groups of actin filaments can specialize in particular structural and motile functions by interacting with one or more of a variety of actin-binding proteins. Little is known of the effects of these proteins on actomyosin activity in nonmuscle cells. Stereociliary actin filaments are extensively cross-linked by fimbrin (14–18), an actin-bundling protein first identified in microvilli and microspikes (19–21). It was not clear whether this would inhibit the myosin movement proposed to mediate adaptation.

To address this question, we modified existing *in vitro* assays for myosin (22, 23), incorporating differential interference contrast (DIC) optics, "optical tweezers" (24–27), and stereociliary cores as the actin substrate. Actin cores were obtained by demembranation of hair bundles (28) isolated by the "bundle blot" procedure (17), preparations that have been studied ultrastructurally and biochemically. Optical tweezers use an infrared laser beam directed through the microscope to capture and manipulate objects by means of radiation pressure—in this case, silica beads coated with myosin thick filaments. We found that stereociliary actin cores were a competent substrate for myosin-based motility, whereas kinociliary cores, containing microtubules, were a competent substrate for kinesin-based motility. Thus, a myosin-like motor remains an attractive mechanism for adaptation in hair cells.

MATERIALS AND METHODS

Blotting and Demembranation of Stereocilia. Bullfrogs (*Rana catesbeiana*) were purchased from Ming's Market (Boston). They were pithed and decapitated, and their sacculi and semicircular canals were removed. Dissection was performed in a cold saline solution (100 μM Ca^{2+}/120 mM NaCl/2 mM KCl/3 mM dextrose/5 mM Hepes, pH 7.2) with protease inhibitors (0.15 μM aprotinin/20 μM leupeptin/0.15 μM pepstatin/0.15 mM phenylmethylsulfonyl fluoride; Boehringer Mannheim). The apical surfaces of the sensory epithelia were exposed by mechanical removal of the oto-

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Abbreviation: DIC, differential interference contrast.