Actin cores of hair-cell stereocilia support myosin motility
(adaptation/cytoskeleton/optical tweezers/auditory system/vestibular system)

GORDON M. G. SHEPHERD*†, DAVID P. COREY*†, AND STEVEN M. BLOCK‡§
*Neuroscience Group, Howard Hughes Medical Institute, and Department of Neurology, Massachusetts General Hospital, Boston, MA 02114; †Program in Neuroscience, Harvard Medical School, Boston, MA 02115; ‡Rowland Institute for Science, 100 Cambridge Parkway, Cambridge, MA 02142; and §Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

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 retoned. 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 CaCl2/120 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-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
lithic membrane or cupula. Stereocilia were isolated by blotting lightly onto coverglasses, which had been cleaned with ethanolic KOH and treated for 1–2 min with poly-L-lysine or poly-L-ornithine (0.1–1.0 mg/ml in H2O; 130–350 KDa; Sigma).

Isolated stereocilia were demembranated in a 1% solution of Triton X-100 in motility buffer (50 mM KCl/25 mM imidazole/4 mM MgCl2/1 mM EGTA, pH 7.4) for 5 min and then washed in motility buffer. Residual adhesive sites on the glass were blocked with bovine serum albumin (0.5 mg/ml in motility buffer; ICN). For fluorescence microscopy, filamentous actin was labeled with Bodipy phallacidin (50 units/ml; Molecular Probes) as described in ref. 17.

**Beak Preparation.** Chicken skeletal myosin was prepared according to ref. 29 and stored at −20°C in 0.5 M KCl/10 mM imidazole, pH 7.0/10 mM EDTA/0.1 mM dithiothreitol/50% (vol/vol) glycerol. All other myosin solutions contained 1 mM dithiothreitol. All solutions were formulated with ionic strength (25 or 50 mM KCl/10 mM imidazole, pH 7.2) and adsorbed onto Covaplates (1.0-μm diameter; Duke Scientific, Palo Alto, CA) or silica beads (0.4-μm diameter silica, prepared by Howard Berg by the procedure given in ref. 30).

The ability of each myosin preparation to support movement was confirmed with the Nitszka assay (22, 23). For assays with optical tweezers and stereociliary cores, it was important for beads to be monodisperse and not clustered in the large aggregates typically observed in the Nitszka assay. This was achieved by using a 1:10 dilution of silica beads incubated with a 1:100 dilution of thick filaments for 10–30 min on ice, with gentle agitation. Bead solutions were triturated before and after dilution. The myosin-coated beads were then diluted 1:4 in motility buffer with 1 mM ATP and 0.5 M sucrose for use in the assay.

Kinesin-coated beads were similarly prepared by coating 0.4-μm-diameter silica beads with squid optic-lobe kinesin (31). Kinesin was prepared as described in ref. 32.

**Chamber Construction.** The assay chamber was made from a 0.5-mm-thick stainless steel slide with a 16-mm-diameter hole in its center. A circular coverglass carrying demembranated cores was affixed with Apiezon M grease (VWR Scientific) to the underside of the slide and centered over the hole, with care being taken to keep the experimental preparation immersed in buffer. A Teflon spacer ring (16-mm i.d.; 1.0-mm thick) was mounted with grease to the top of the slide, over the hole (giving a chamber vol of 300 μl), and motility buffer with 1 mM ATP was added. To assay motility, 1–3 μl of the bead preparation was introduced into the chamber for which was then sealed by mounting a coverglass to the top.

**Optical Tweezers.** A single-beam gradient-force optical trap (optical tweezers) was created by passing the beam from a continuous-wave Nd:YAG laser (output power, 1 W; TEM00 mode; wavelength, 1064 nm; C-95 YAGMAX, CVI/Laser, Albuquerque, NM) through the objective of an inverted microscope. Salient features of the design may be found in ref. 27. The x–y–z position of the trapping zone in the microscope was controlled by means of external optics; the diameter of the trapping zone was fixed at ≈1 μm, corresponding to the diameter of a diffusion-limited spot. The system was equipped with a variable attenuator and shutter to adjust the trapping force and to release the trap, respectively. Trapping force was calibrated against Stokes’ drag (26). Trap alignment and position were monitored by imaging the laser spot, arising from partial reflection of the light at the coverglass-water interface, on a video camera sensitive to the near infrared (extended-red Ultracon tube, series 75; Dage-MTI, Michigan City, IN). The same video camera was used to observe the microscope image at visible wavelengths.

**Imaging.** Fluorescence microscope slides were stained with oil-immersion condenser (numerical aperture (n.a.), 1.4) and oil-immersion objective (ICS 100X Plan-Neofluar; n.a., 1.3; Zeiss) on an inverted microscope equipped with DIC optics (Axiovert 35; Zeiss); this same objective served to form the optical trap. Images were taken by video camera and stored with a videocassette recorder (AG-6300; Panasonic, VHS format). Bead speeds were measured by using a semiautomatic computer-based system that acquires bead position and video frame count (33). For high-resolution tracking of bead movements, video images were first transferred to an optical memory disk recorder (TO-2026F; Panasonic), and then analyzed by software that performs centroid analysis of the bead image on a frame-by-frame basis using a cross-correlation algorithm (Image-1, Universal Imaging (Media, PA) and custom software; for methods, see ref. 34). Bead coordinates were fit to a linear trajectory that minimized the least-squares perpendicular distance of each point to the line. Centroid analysis gives subpixel resolution, corresponding to positional errors of ≈20 nm for the bead sizes and magnifications used in this study.

**RESULTS**

**Actin Cores.** To monitor the core preparation procedure, blotted and demembranated stereocilia were labeled with a fluorescent actin probe (Fig. 1). They appeared as hundreds of long straight rods over an area of 0.5–1 mm². Treatment of the coverglass with poly-L-ornithine or poly-L-lysine increased the yield of the isolation technique manifold over blotting onto untreated glass. Contamination by cuticular plates and zonulae adherentes, the other actin-containing organelles at the hair-cell apical surface, was minimal. When the same fields were imaged with DIC optics, the stereocilia were seen to be straight rods ≈0.4 μm wide and 50–100 μm long. Often, stereociliary polarity could be discerned by the basal tapering and blunt tip, or by the geometry of a blotted bundle, in which tips tended to stay together and bases tended to splay apart. Kinocilia were identified by their characteristic curved appearance, smaller diameter, and often the presence of the kinociliary bulb (Fig. 1c).

**Myosin Motility.** Myosin-coated beads at low density, diffusing through the medium, took many minutes to reach the cores. In contrast, micromanipulation of individual beads with optical tweezers enabled direct placement on selected
cores in a matter of seconds. Beads reaching the cores by either method bound and moved for distances of 1–10 μm. Fig. 2a shows a bead being applied to a semicircular canal core by optical tweezers, then binding, escaping the laser trap, and moving several micrometers along the core. This escape was anticipated; our optical tweezers produced ≈10 pN of force on a 0.4-μm-diameter silica bead, while a thick filament propelling a bead may have 10–100 active myosin heads, each producing 1–10 pN of force (35).

With optical tweezers, the substrate specificity of mechanoenzyme-coated beads was readily tested. Kinesin-coated beads, deposited by optical tweezers on kinociliary cores, moved at rates of 0.1–0.2 μm/s (Fig. 2b), similar to rates obtained with the same kinesin preparation on sea urchin axonemes (26). Kinesin-coated beads moved only on kinociliary cores, and myosin-coated beads moved only on stereociliary cores.

Bead movement could not be attributed to any stereociliary mechanoenzymes. Beads that moved did so at rates characteristic of chicken skeletal muscle myosin, while beads coated either with kinesin or with small amounts of myosin at concentrations below the threshold for movement, failed to pick up endogenous motors and move on stereociliary cores.

Bead movements were unidirectional, toward what were identified as the tips of the stereocilia. Fig. 3 shows three beads that moved along stereociliary tracks, joining previously accumulated beads near the top end of the bundle. Beads that moved to the ends of cores always remained bound, not releasing from the actin. The reason for this binding was unclear. Possibly, it involved other structures at the tips of stereocilia.

The speed of myosin-coated beads moving on cores averaged 1–2 μm/s, the same range as speeds obtained with the same myosin preparation in Nitella assays (data not shown). Speeds were the same whether beads reached the cores by diffusion alone or were delivered by optical tweezers, suggesting that the laser light did nothing to impair myosin motility. Once moving, a motile bead did not appear to be halted or even slowed by optical tweezers. Some beads exhibited smooth, uniform movement, while others slowed or stopped briefly.

The fine structure of the motion was analyzed by frame-by-frame centroid tracking of bead position. The myosin-coated bead of Fig. 2a moved in a nearly straight trajectory along the core (Fig. 4a). A plot of the component of this motion parallel to the trajectory against time showed variation in the speed of the bead (Fig. 4b). It paused briefly, ran for a stretch with some variation in speed (peak speed, 1.5 μm/s), then paused and started again. A plot of the motion perpendicular to the trajectory against time showed only minimal side-to-side deviations (10–50 nm), until the final 0.5 s, when the direction changed slightly (Fig. 4c).

![Fig. 2.](image-url) (a) A myosin-coated bead (arrow) was deposited by optical tweezers on a stereociliary core. Successive images (top to bottom) show the bead moving out of the trap (seen as the bright spot in the first three images) and along the core for several micrometers, at a speed of ≈1.5 μm/s. The bead eventually stopped near the final position shown. (b) A kinesin-coated bead (arrow) was deposited by the optical tweezers on a kinociliary core. Successive images (top to bottom) show the bead moving along the core for a distance of several micrometers, at a speed of ≈0.1 μm/s. The trap can be seen picking up another bead (bright spot) in the final two images.

![Fig. 3.](image-url) Three myosin-coated beads (arrows) moved from the bases (a) to the tips (b) of sacculcular stereociliary cores, joining clusters of beads already present at the tips.
the forces thought to be generated by a single myosin head (35).

The polarity of movement is also consistent with the model. Myosins move unidirectionally on actin filaments, toward their barbed ends (36). The actin filaments of stereocilia are uniformly oriented with their barbed ends at the tips (16, 28, 37). A myosin moving on stereociliary actin filaments would therefore be expected to climb toward the tip, as we have observed here; this is the direction required for tensioning in the hair cell adaptation model.

Microvilli of the intestinal brush border also have a polar actin core, laterally linked to the membrane by a myosin (38, 39). This brush border myosin I (38), unlike skeletal muscle myosin and other myosins II, resembles other myosins I (reviewed in ref. 40) in having a short tail. This tail is apparently specialized for functions other than filament formation, such as binding to membranes. Brush border myosin I also binds calmodulin (41) and displays Ca\(^{2+}\)/calmodulin-dependent motility on actin in vitro (38, 39). Although its physiological role is currently unknown, the presence of a myosin in a structure so similar to stereocilia is intriguing. However, brush border myosin I itself does not seem to be the actin-to-membrane linker of stereocilia. Gels of purified stereociliary cores lack a band near the expected mass of 110 kDa (17). Brush border myosin I requires ATP for elution from demembranated microvillar cores (42), but the stereocilia actin-to-membrane linkers do not (17, 28).

Evidence for other myosins in stereocilia is equivocal. Labeling of stereocilia with antibodies to smooth muscle myosin has been reported (43) but disputed on the basis of light-piping artifacts (44) and on the failure of labeling by several anti-myosin antibodies (45). However, the latter results may simply reflect a lack of antibody cross-reactivity among myosins. Inasmuch as the adaptation motor may be present in hair bundles at a concentration of only a few molecules per stereocillum, conventional gel and immunocytochemical methods may be inadequate for detection. It is interesting to note, however, that calmodulin appears localized to the tips (17).

Our results show that stereociliary actin is available as a substrate for myosin motility and thereby provide circumstantial evidence supporting the proposal that an actin-based motor is present in stereocilia.

**Fimbrin and Myosin Motility.** Some actin-binding proteins inhibit actin–myosin interactions. The 55-kDa protein that bundles actin filaments, actin-severing proteins, and the 22-kDa subunit of villin prevents binding of myosin S-1 fragments to the actin bundle (46). Villin, at certain concentrations, inhibits actomyosin ATPase activity (47). However, other observations indicate that myosin motility is compatible with the presence of some actin-binding proteins. Niella actin filaments, bundled into cables by an uncharacterized protein, support myosin motility (22, 23), as do actin filaments bound by phalloidin (13). The fimbrin-bundled actin cores of microvilli and stereocilia can be decorated by myosin S-1 fragments (and by brush border myosin I, in the case of microvilli) but myosin motility along these cores has not been previously reported (37, 48).

Our results show that myosin can bind and move along stereociliary actin filaments in the presence of actin-binding proteins. The actin cores of saccular stereocilia have a relatively simple biochemical composition, consisting primarily of actin and fimbrin, with several other proteins present in lower abundance (17). We cannot rule out the possibility that myosin movement in our assay was along bare actin filaments that had become partially dissociated from the cores. However, the ionic conditions favored the association of fimbrin with actin (20, 21, 28), and the cores, in addition to containing fimbrin when assayed electrophoretically (17),

**DISCUSSION**

**An Actin-Based Motor in Stereocilia?** Several lines of evidence implicate a myosin-like motor in hair cell adaptation. Measured physiologically, the rate of reorientation after a hyperpolarizing bundle displacement is relatively constant. The reorientation rate can be accounted for by movement of the attachment point of the tip linkage upward, along the side of a stereocillum, at a speed of 1–2 μm/s (10), comparable to the speeds of myosins. The forces required to open the transduction channels, determined from measurements of bundle stiffness, are in the 1–5 pN range (1), comparable to

![Diagram](image-url)
did not have the characteristic, curved morphology of “de-
embrinated” stereociliary cores (28).

A Myosin Assay. In addition to demonstrating the move-
ment of myosin along stereociliary actin cores, these ex-
periments illustrate the application of two useful technologies to in
vitro motility assays for myosin. Using optical tweezers as a
micromanipulator for bead delivery improved the efficiency of the
assay, enabling precise placement (and release) of beads onto the
substrate. This feature has recently been exploited in motility assays for kinesin to study movement based on single kinesin molecules (31). An optically thin preparation of actin bundles that allowed video-enhanced DIC optics facilitated precise measurements of position: good spatial and temporal resolution are more easily achieved with refractile beads and DIC optics than with fluorescent
actin filaments and low light-level cameras (e.g., see ref. 13). DIC
optics were previously used to visualize endogenous motor activity on ChvA
actin cables by Kachar (49). We anticipate that the combination of these technologies, coupled
with the relatively simple biochemical components of this assay, will prove helpful in the further study of actin-
based motors.

We thank Bruce Schnapp for advice and help with kinesin assays and tracking analysis. Dan Kiehart and Tung-Ling Cheng generously provided lab space and advice for myosin purification. Howard Berg kindly donated silica beads. We thank Bechara Kachar for suggesting poly-L-ornithine and for stimulating our interest in motility assays.
This work was supported by grants from the National Institutes of Health (NS-22059) and the Office of Naval Research (754-9002-4) to D.P.C., and by the Rowland Institute for Science (S.M.B.). D.P.C. is an Associate Investigator of the Howard Hughes Medical Institute.

7.
Res. 15, 103–112.
USA 84, 3064–3068.
961.
7, 2821–2836.
Neurosci. 9, 3988–3997.
Acad. Sci. USA 86, 2918–2922.
83, 6272–6276.
86, 244–250.
Natl. Acad. Sci. USA 86, 4973–4977.
18. Tilney, M. S., Tilney, L. G., Stephens, R. E., Merte, C.,
Cell Biol. 109, 1711–1723.
J. Biol. Chem. 256, 2983–2988.
6853.
31–35.
99, 1867–1871.
(London) 330, 769–771.
don) 338, 514–518.
27. Block, S. M. (1990) in Noninvasive Techniques in Cell Biology,
375–401.
Acta 41, 401–421.
30. Stoeber, W., Fink, A. & Bohn, E. (1968) J. Colloid Interface
(London) 331, 450–453.
533–538.
2395–2400.
110, 1137–1147.
Biophys. Chem. 17, 23–45.
10554.
667–673.
(London) 288, 491–492.
45. Drenckhahn, D., Kellner, J., Mannherz, H. G., Grosse-
(London) 300, 531–532.
1850–1857.