ATPase Activity of Myosin in Hair Bundles of the Bullfrog's Sacculus

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ABSTRACT Mechanoelectrical transduction by a hair cell displays adaptation, which is thought to occur as myosin-based molecular motors within the mechanically sensitive hair bundle adjust the tension transmitted to transduction channels. To assess the enzymatic capabilities of the myosin isozymes in hair bundles, we examined the actin-dependent ATPase activity of bundles isolated from the bullfrog's sacculus. Separation of ^{32}P-labeled inorganic phosphate from unreacted [γ-^{32}P]ATP by thin-layer chromatography enabled us to measure the liberation of as little as 0.1 fmol phosphate. To distinguish the Mg^{2+}-ATPase activity of myosin isozymes from that of other hair-bundle enzymes, we inhibited the interaction of hair-bundle myosin with actin and determined the reduction in ATPase activity. *N*-ethylmaleimide (NEM) decreased neither physiologically measured adaptation nor the nucleotide-hydrolytic activity of a 120-kDa protein thought to be myosin Iβ. The NEM-insensitive, actin-activated ATPase activity of myosin increased from 1.0 fmol $\times$ s^{-1} in 1 mM EGTA to 2.3 fmol $\times$ s^{-1} in 10 μM Ca^{2+}. This activity was largely inhibited by calmidazolium, but was unaffected by the addition of exogenous calmodulin. These results, which indicate that hair bundles contain enzymatically active, Ca^{2+}-sensitive myosin molecules, are consistent with the role of Ca^{2+} in adaptation and with the hypothesis that myosin forms the hair cell's adaptation motor.

INTRODUCTION

Throughout the vertebrates, sound and acceleration are detected by hair cells located in the auditory and vestibular organs of the internal ear (reviewed in Hudspeth, 1989). The most striking morphological characteristic of a hair cell is the presence on its apical surface of a hair bundle comprising several stepped rows of actin-filled stereocilia and a single kinocilium. Deflection of a hair bundle in the positive direction, toward its tall edge, depolarizes the cell, increases neurotransmitter release, and thus excites postsynaptic activity in afferent nerve fibers; bundle movement in the opposite direction leads to a hyperpolarization and a reduction in neural activity. These electrical responses are initiated by the flow of a cationic current, dominated by K^+ but also including Ca^{2+} (Corey and Hudspeth, 1979; Ohmori, 1985), through mechanosensitive ion channels located near the stereociliary tips (Hudspeth, 1982; Jaramillo and Hudspeth, 1991; Denk et al., 1995; Lumpkin and Hudspeth, 1995). The channels are thought to be gated by the tension in fine filaments, known as tip links, that interconnect adjacent stereocilia (Pickles et al., 1984; Furness and Hackney, 1985). Positive stimulation increases the tension in these tip links and thereby opens transduction channels; bundle deflection in the negative direction reduces tip-link tension and diminishes the current flowing at rest.

When a hair bundle is subjected to a prolonged deflection of either polarity, the transduction process adapts: the transduction current returns to near its resting level within a few tens of milliseconds, and subsequent stimulation reveals the bundle to be fully sensitive to displacement from its new holding position (Eatock et al., 1987). This form of adaptation confers extraordinary sensitivity upon hair cells: by continuously resetting the transduction apparatus for maximal responsiveness, these cells can detect transient stimuli more than six orders of magnitude smaller than the background input (Narins and Lewis, 1984).

It is believed that adaptation is mediated by molecular motors that adjust tip-link tension by moving each link's attachment point, the insertional plaque, up or down a stereocilium (Howard and Hudspeth, 1987a,b; Assad and Corey, 1992; reviewed in Hudspeth, 1989; Hudspeth and Gillespie, 1994). The sensitivity of adaptation to ADP and its analogs (Gillespie and Hudspeth, 1993) implies the participation of motor molecules fueled by ATP. Because extracellular Ca^{2+} affects the rate of adaptation (Eatock et al., 1987; Hacohen et al., 1989) and calmodulin inhibitors block the process (Walker and Hudspeth, 1996), the adaptation motor is probably regulated by the binding of Ca^{2+} to calmodulin, a protein that occurs at a high concentration in hair bundles (Shepherd et al., 1989; Walker et al., 1993). This control by calmodulin, as well as the preponderance of actin in stereociliary cores, implicates myosin as a constituent of the adaptation motor. Moreover, the rate of adaptation (Howard and Hudspeth, 1987a; Assad and Corey, 1992) and the forces involved (Jaramillo and Hudspeth, 1993) are consistent with values reported for myosin molecules (reviewed in Hudspeth and Gillespie, 1994).

Of the several myosin isozymes expressed in hair cells (Metcalf et al., 1994; Solc et al., 1994; Avraham et al., 1995), immunofluorescence microscopy situates at least myosin Iβ, myosin VI, and myosin VIIa in hair bundles (Gillespie et al., 1993; Hasson et al., 1995; personal communication, D. P. Corey, Massachusetts General Hospital, Boston, MA). Myosin VI and myosin VIIa are clearly of

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importance in hair-cell function, for mutation of their corresponding genes causes hereditary deafness due to hair-cell degeneration (Avraham et al., 1995; Gibson et al., 1995; Weil et al., 1995). Which of these—or perhaps other—myosin isozymes is responsible for adaptation remains uncertain. Two circumstantial arguments favor myosin Iβ as the motor molecule for adaptation. Unlike myosins VI and VIIa, myosin Iβ is concentrated near stereociliary tips (Gillespie et al., 1993), the site of adaptation (Assad et al., 1989). In addition, photolabeling with nucleotides in the presence of vanadate reveals that the 120-kDa myosin Iβ is enzymatically active.

Although these results implicate myosin Iβ as the motor molecule responsible for adaptation, the ATPase activity of the myosin in hair bundles has not been directly measured. The small number of myosin molecules in each hair bundle, about 10^4 in the instance of myosin Iβ (Gillespie et al., 1993; Walker and Hudspeth, 1996), precludes both the use of standard biochemical techniques to purify specific myosin isozymes and the application of conventional ATPase assays to determine the rate of ATP hydrolysis. We have therefore developed a new method based on the separation of ^{32}P-labeled phosphate from [γ-^{32}P]ATP by thin-layer chromatography (TLC) and subsequent detection on phosphor storage screens. This assay has enabled us to detect the ATPase activity of the myosin molecules in the hair bundles isolated from a few bullfrog sacculi. By using this assay, we have also explored the sensitivity of hair-bundle myosin ATPase activity to conditions known to affect adaptation. The results provide the first estimates of the ATPase activity of an unconventional myosin isozyme in its native environment.

MATERIALS AND METHODS

Agarose (SeaPlaque GTG or SeaKem LE) was obtained from FMC BioProducts (Rockland, ME), [γ-^{32}P]ATP from Amersham Corporation (Arlington Heights, IL), calmidazolium chloride from Fluka Chemical Corporation (Ronkonkoma, NY), Cell-Tak adhesive from Becton Dickinson Labware (Bedford, MA), $KH_2[^{32}P]O_4$ from DuPont NEN Research Products (Boston, MA), and sodium orthovanadate from Fisher Scientific (Pittsburgh, PA). The protease inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), leupeptin, and pepstatin were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). Rabbit-muscle actomyosin and its proteolytic product S1, a kit for the molybdate-based colorimetric assay of inorganic phosphate, and the chemicals for which no origin is indicated were obtained from Sigma Chemical Company (St. Louis, MO).

Hair-bundle preparation

Sacculi were dissected from the internal ears of adult bullfrogs (*Rana catesbeiana*) and placed in saline solution containing 110 mM Na^+, 2 mM K^+, 4 mM Ca^{2+}, 118 mM Cl^-, 3 mM D-glucose, and 5 mM *N*-(hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid (HEPES) at pH 7.3. To retard proteolysis, the solution was supplemented with 200 μM AEBSF, 1 μM leupeptin, and 1 μM pepstatin. This and other solutions used during dissection and bundle isolation were maintained at 4°C.

Hair bundles were isolated by the twist-off method (Gillespie and Hudspeth, 1991). Dissected bullfrog saccular maculae were attached, with their hair bundles facing upward, to a coverslip coated with Cell-Tak; the otolithic membranes were then removed with forceps. A 3% solution of agarose at 37°C was poured over the preparation and allowed to solidify at 4°C. When the gelled agarose was twisted free of the sacculi, the hair bundles were sheared from the cells' apical surfaces and remained embedded in the gel. Small pieces of agarose containing isolated hair bundles were removed and frozen in liquid nitrogen. Samples were ordinarily used in experiments shortly after preparation, but were occasionally stored at −80°C for no longer than 1 week.

To permit quantitative assessments of ATPase activity under various circumstances, we normalized the results to saccular equivalents (SE) of hair bundles (Gillespie and Hudspeth, 1991). A saccular equivalent contains ~2500 hair bundles, each of which comprises ~50 stereocilia; the total protein content of a saccular equivalent is ~40 ng.

ATPase assay

The hydrolysis of ATP by isolated, permeabilized hair bundles was monitored by the use of TLC to separate radiolabeled inorganic phosphate (P_i) from unreacted [γ-^{32}P]ATP. Samples to be tested were applied to basic ion-exchange TLC plates (Polygram Ionex-25 SA-Na, Bodman, Aston, PA) and their components resolved by migration for 10–15 min in 25 mM HCl. The plates were then wrapped in clear plastic sheets and placed against phosphor storage screens for 1–12 h. Exposed screens were examined with a storage phosphor imager (PhosphorImager 400, Molecular Dynamics, Sunnyvale, CA).

Areal integration of rectangles including each migration lane (ImageQuant 3.3, Molecular Dynamics) resulted in two clearly resolved peaks, one for the inorganic phosphate migrating near the solvent front, the other for ATP remaining near the application point. Assuming that the fraction, f, of the total signal that was found in the phosphate peak reflected the fraction of the total ATP that was hydrolyzed in the sample, the ATPase activity was calculated as

$$V_{\mathrm{ATPase}} = \frac{(f - f_0)M_{\mathrm{ATP}}}{Nt},$$

in which f_0 is the fraction of the total signal in the phosphate peak in the absence of enzyme, M_{ATP} is the total amount of ATP present in the reaction, N is the number of saccular equivalents, and t is the time of incubation.

The sensitivity of this method resulted primarily from the small volumes that sufficed in the separation of inorganic phosphate from ATP. Although 1-μl aliquots were ordinarily used as TLC samples, volumes as small as 0.2 μl gave similar results. Depending upon the storage time of the [γ-^{32}P]ATP, f_0 ranged from 0.004 to 0.008. To ensure adequate detectability and a linear response, the value of f was maintained in the interval 0.020–0.100; the dynamic range of the method could then be optimized by adjusting the value of M_{ATP}, provided that the concentration remained at least twice the relevant enzyme's Michaelis constant (K_{M}).

We performed a control experiment to confirm the linearity of the phosphor-screen imaging used to measure radioactivity. After the application to a TLC plate of 1-μl samples of [γ-^{32}P]ATP with activities of 0.075–37 Bq, a phosphor storage screen was exposed to the plate for ~1 h. The screen was then scanned and intensities were determined by areal integration. We found that the ^{32}P signal measured with a storage phosphor imager was linearly related to the amount of radiolabel applied over more than two orders of magnitude in the range appropriate for our experimental measurements (data not shown).

Total ATPase activity

Approximately three saccular equivalents of isolated hair bundles, still embedded in agarose, were used in each sample. Stereociliary membranes were permeabilized by a 15-min incubation at 4°C with 40 mg × l^{-1} saponin in Mg^{2+}-ATPase assay solution, which contained 25 mM KCl, 1

mM $MgCl_2$, 1 mM EGTA, and 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris) at pH 7.3. When appropriate, pretreatment reagents such as thapsigargin, ouabain, and NEM were added to this permeabilization solution. Samples were then sedimented by centrifugation and the solution was replaced with the Mg^{2+}-ATPase assay solution. Mg^{2+}-ATPase assay reactions were run at room temperature, ~22°C, usually for 30 min in a volume of 6–10 μl. The free Ca^{2+} concentration throughout the assay was generally adjusted to 10 μM by the addition of $CaCl_2$; when specified, the free Ca^{2+} concentration was instead made negligible or set at 0.1 μM, 1 μM, 10 μM, or 100 μM with 1 mM EGTA (Marks and Maxfield, 1991). K^+-EDTA ATPase activity was measured after incubation for 30–60 min at room temperature in 5 mM EDTA, 600 mM KCl, and 25 mM Tris at pH 7.3. The concentration of ATP used, 100 μM, was measured spectrophotometrically using an extinction coefficient at 260 nm of $1.54 \times 10^6\ M^{-1} \times m^{-1}$. To each sample was added 9.25 kBq of [γ-^{32}P]ATP at a specific activity of 370 MBq × mol^{-1}. A Lineweaver-Burk plot of K^+-EDTA ATPase values, measured at ATP concentrations of 0.1–100 μM, yielded a K_M value of 30 μM.

Myosin ATPase activity

Twist-off preparations of hair bundles contain ATPases in addition to myosin. The contribution of these contaminants was reduced with specific inhibitors, such as 15 μM thapsigargin for the Ca^{2+} pump (or Ca^{2+}-ATPase) and 100 μM ouabain for the Na^+ pump (or Na^+/K^+-ATPase). Where indicated, 320 μM NEM was used to inhibit ATPases with cysteinyl residues at their active sites.

Because the microfilament cores of stereocilia remain intact following the twist-off procedure and exposure to solutions of moderate ionic strength (Gillespie and Hudspeth, 1991), we relied upon hair bundles' endogenous actin to activate myosin molecules in our assays. Stereocilia contain a high actin concentration, about 2 mM for the bullfrog's saccular hair cell. Actin-dependent ATPase activity was defined as that portion of a sample's activity lost when interaction between myosin and actin was inhibited. After the total ATPase activity had been measured, hair bundles in agarose were sedimented for 30 s in a centrifuge. To saturate the myosin-binding sites on actin filaments, we then replaced the ATPase solution with 0.16 g × l^{-1} of *N*-ethylmaleimide-treated subfragment 1 from myosin II (S1-NEM; Borejdo and Burlacu, 1992) in assay solution and maintained the bundles for 45 min at 0°C. Unbound S1-NEM and unmodified S1 were removed by two 15-min washes, the first in assay solution supplemented with 100 μM ATP and the second in assay solution alone. The ATPase activity was then measured again and the difference between the two values was attributed to myosin.

In control experiments, we ascertained that repeated incubations in the absence of S1-NEM resulted in little or no change in either the Mg^{2+}-ATPase or the K^+-EDTA ATPase activity. On a representative experiment, the Mg^{2+}-ATPase of a sample declined by less than 5% during four successive assays. In keeping with previous observations (Gillespie et al., 1993), this result indicates that the myosin molecules contributing to the measured ATPase activity are not solubilized by our protocol.

In an alternative procedure, the interaction of myosin with actin was prevented by use of either of two peptides that mimicked parts of the actin-myosin interaction surface. The peptide Myl (KFKGIISILDEECLRPGEATDLTFL) reproduces amino-acid residues 463–487 of mammalian myosin Iβ (Reizes et al., 1994), which differs from amphibian myosin Iβ by only one residue in this region (Metcalf et al., 1994; Solc et al., 1994). As a control, experiments were also performed with a scrambled version of this molecule (DKLFIKSGIITEAECEGLPRLFTLD; Biopolymer Facility, University of Texas Southwestern Medical Center, Dallas, TX). The peptide Acl (DETQDTALVCDD; Protein/DNA Technology Center, The Rockefeller University, New York, NY) represents the negatively charged amino terminus of actin (Elzinga et al., 1973). Peptide was added to a Mg^{2+}-ATPase reaction at a concentration of 0.5 g × l^{-1} for Myl or 1 g × l^{-1} for Acl.

In a control experiment, the actin-activated ATPase activity of recombinant amphibian myosin Iβ was assessed by the technique used to quantitate hair-bundle ATPase activity. Approximately 1 μg of myosin Iβ, partially purified from protein produced in Sf9 insect cells with a baculovirus expression system, was incubated for 30 min at room temperature with 23 μM F-actin and 100 μM ATP in the Mg^{2+}-ATPase assay solution defined above.

In studies of the effect of calmidazolium on myosin's Mg^{2+}-ATPase activity, the total activity was first measured as described, after which the measurement was repeated in the presence of 5 μM calmidazolium chloride. Following S1-NEM treatment, calmidazolium was again added to the assay solution. Because calmidazolium was prepared as a stock solution in dimethyl sulfoxide (DMSO), these assay solutions and the associated controls contained 70 μM DMSO.

Photolabeling of hair-bundle myosin

The relative contributions of various isozymes to hair-bundle myosin ATPase activity were assessed by using a slightly modified technique (Gillespie et al., 1993) to measure the extent of photolabeling with the vandate ion-trapped hydrolysis products of [α-^{32}P]UTP. As described for the ATPase measurements, each sample containing bundles from approximately three saccular maculae was permeabilized and pretreated with 40 mg × l^{-1} saponin for 15 min at 4°C. During this step, some samples were additionally treated with 300 μM NEM. The radiolabeled UDP that resulted from the sample's nucleotide-hydrolytic activity was trapped at myosin's active site by 30-min incubation at room temperature in a solution containing 1–2 μM [α-^{32}P]UTP, 1 mM AMP, 1 mM sodium orthovanadate, 100 mM KCl, 5 mM $MgCl_2$, 20 μM $CaCl_2$, 200 μM AEBSF, 1 μM leupeptin, 1 μM pepstatin, and 25 mM HEPES at pH 7.3. After a 30-min wash with four changes of the same solution lacking nucleotides and vanadate, bundles were irradiated for 10 min at 0°C with 254-nm light. As measured with a short-wavelength light meter (J-225, UVP, Inc., San Gabriel, CA), the intensity of illumination at the samples was 24 W × m^{-2}. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% or 4–12% gels (Novex, San Diego, CA). The gels were silver-stained, dried, scanned with a densitometer (Silverscanner II, LaCie, Ltd., Beaverton, OR), and exposed to phosphor storage screens. In each lane, the radioactivities associated with myosin isozymes of molecular masses near 120 kDa, 160 kDa, and 230 kDa were normalized with respect to the density of the corresponding silver-stained actin band. The change in the labeling of each myosin isozyme produced by NEM was calculated as the difference between the normalized signals for the control and NEM-treated samples, divided by the control value.

Electrophysiological recording

Hair-cell isolation and tight-seal, whole-cell recording were variants of previously published methods (Lumpkin and Hudspeth, 1995). The internal solution contained 100 mM *N*-methyl-D-glucamine, 12 mM Na^+, 1.6 mM Ca^{2+}, 3 mM Mg^{2+}, 98 mM Cl^-, 2 mM ATP, 2 mM 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and 5 mM HEPES at pH 7.3; when noted, 300 μM NEM was added. To elicit transduction currents, hair bundles were subjected to positive and negative displacements ranging from −500 nm to +500 nm in 200-nm steps.

Throughout the text, numerical results from replicated experiments are expressed as means ± standard deviations for the indicated numbers of experiments. Results presented without standard deviations refer to single experiments.

RESULTS

High-sensitivity measurement of ATPase activity

Measuring the ATPase activity of the very limited number of myosin molecules in hair bundles necessitated the devel-

opment of an extremely sensitive assay. By separating phosphate from ATP with TLC (Fig. 1 *A*) and by quantitating the liberation of phosphate with storage-phosphor imaging, we were able to detect ATPase activities as small as 0.1 fmol $\times$ s^{-1} per hair-bundle sample. When mixtures containing known ratios of phosphate and ATP were separated and analyzed, the relation between the predicted and measured values displayed a slope of 0.99 with a correlation coefficient exceeding 0.99 (Fig. 1 *B*). Because the rate of ATP hydrolysis by bundles was nearly linear for at least 45 min (Fig. 1 *C*), all further measurements were based upon 30-min incubations.

We additionally confirmed that our estimates of ATPase activity accorded with those obtained by conventional methods. Quantitation of the ATPase activity of rabbit skeletal-muscle actomyosin by TLC separation yielded a value, 147 $\pm$ 25 nmol $\times$ s^{-1} $\times$ g^{-1} (n = 7), in excellent agreement with that obtained with a colorimetric method, 138 $\pm$ 25 nmol $\times$ s^{-1} $\times$ g^{-1} (n = 3).

ATPase activity of hair-bundle myosin

The total ATPase activity was measured for hair bundles isolated by the twist-off method and still embedded in agarose. The average activity for 17 preparations was 8.2 $\pm$ 2.4 fmol $\times$ s^{-1} $\times$ SE^{-1}, that is, per saccular equivalent of hair bundles. Each saccular equivalent, which contains ~40 ng protein, includes ~2,500 hair bundles, each comprising some 50 stereocilia. The measured activity therefore corresponded to ~200 nmol $\times$ s^{-1} $\times$ g^{-1}, to 2 amol $\times$ s^{-1} per hair bundle, or to 50 zmol $\times$ s^{-1} per stereocilium. These values included the activities of the putative adaptation motors, of the additional myosin isozymes in stereocilia, and of other ATPases in hair-bundle preparations.

The contribution of NEM-insensitive forms of myosin to total bundle ATPase activity was estimated in four independent ways. Because the mechanoenzyme constituting the adaptation motor has not been identified with certainty, none of these methods yielded an unequivocal measure of the motor's activity. The results, which are summarized in Table 1, nonetheless provided a consistent estimate of the activity of myosin isozymes whose properties are compatible with those of the motor.

The first approach used pharmacological agents to inhibit ATPases other than the adaptation motor. Thapsigargin was used to inactivate Ca^{2+} pumps in the endoplasmic reticulum (Takemura et al., 1989); ouabain was used to inhibit the Na^+ pump of the basolateral cellular surface. NEM, a derivatizing reagent for cysteinyl residues, served as a nonspecific inhibitor of numerous ATPases, including the plasmalemmal Ca^{2+} pump (Carafoli, 1991), the Na^+ pump (Richards et al., 1977), and kinesin (Wagner et al., 1989). After hair-bundle preparations had been exposed to thapsigargin, ouabain, and NEM, the remaining ATPase activity averaged 2.1 $\pm$ 0.5 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 5).

NEM also inhibits myosin II (Pemrick and Weber, 1976) and perhaps other myosin isozymes with cysteinyl residues at the vulnerable SH1 or SH2 positions of their active sites. To determine whether NEM affects the motor molecules underlying adaptation, we performed physiological experiments on hair cells isolated from the bullfrog's sacculus. When cells were dialyzed with an internal solution containing 300 μM NEM, adaptation to both positive and negative stimuli generally persisted for as long as in recordings from control cells (Fig. 2). For four hair cells that displayed robust responses, the magnitudes of transduction currents, timecourses of adaptation, and durations of responsiveness (397 s, 511 s, 810 s, and 1048 s) were comparable to those of control cells dialyzed with an internal solution lacking NEM. Adaptation of the mechanoelectrical-transduction process therefore appears relatively insensitive to NEM.

Although NEM reduced the total ATPase activity of hair bundles by 30–50%, the reagent had no significant effect on the activity of 120-kDa hair-bundle myosin, as estimated from the intensity of the protein's photolabeling with the vanadate-trapped hydrolysis products of [α-^{32}P]UTP (Fig. 3). In six experiments, 300 μM NEM changed the intensity of photolabeling of the 120-kDa myosin isozyme by 0 $\pm$

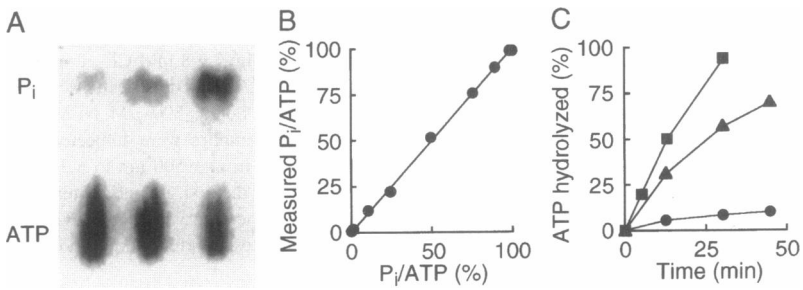

FIGURE 1 Measurement of myosin's ATPase activity by separation of inorganic phosphate (P_i) from ATP with TLC and scanning of phosphor storage screens. (*A*) During separation by TLC, hydrolyzed phosphate migrated with the solvent front near the figure's top, whereas unreacted ATP lagged behind near the application points. In this control experiment, inorganic phosphate resulted from the ATPase activity of 0.1 g $\times$ l^{-1} rabbit skeletal-muscle actomyosin for (from *left* to *right*) 0 min, 1 min, or 15 min. (*B*) When mixtures of $KH_2[^{32}P]O_4$ and [γ-^{32}P]ATP were applied to a TLC plate, separated, and quantitated, the measured ratios of inorganic phosphate to ATP closely matched the expected values. The r^2 value for a least-squares fit was 0.99. (*C*) The time course of ATP hydrolysis is shown for 0.1 mg of glycerinated frog leg muscle (*squares*), residual saccular maculae left after hair-bundle removal (*triangles*), and isolated saccular hair bundles (*circles*). The total ATP concentration was initially 100 μM in each instance.

TABLE 1 ATPase activity measured in hair bundles

Description of ATPase activity	Activity and SD (fmol × s^{-1})	No. of observations
Mg^{2+}-ATPase	8.2 ± 2.4	17
Myosin Mg^{2+}-ATPase*	5.0	1
NEM-insensitive myosin Mg^{2+}-ATPase#	2.2 ± 0.5	13
Ca^{2+}-free medium	1.0 ± 0.6	5
NEM-insensitive myosin Mg^{2+}-ATPase*	1.8 ± 1.0	3
NEM-insensitive myosin Mg^{2+}-ATPase§	1.6 ± 0.4	4
NEM-insensitive myosin Mg^{2+}-ATPase¶	2.4 ± 0.9	9
plus calmidazolium	0.8 ± 0.2	3
plus calmidazolium and calmodulin	2.6 ± 0.2	2
K^{+}-EDTA ATPase	0.4 ± 0.2	27

Each value refers to the ATPase activity attributed to one saccular equivalent of hair bundles. As described in the text, the actin-dependent Mg^{2+}-ATPase activities of myosin were isolated by several treatments:
*Inhibition with the peptide My1.
#Inhibition of competing ATPases with thapsigargin, ouabain, and NEM.
§Inhibition with the peptide Ac1.
¶Inhibition with S1-NEM.
All measurements except those in Ca^{2+}-free medium and the last were conducted in the presence of 10 μM free Ca^{2+}.

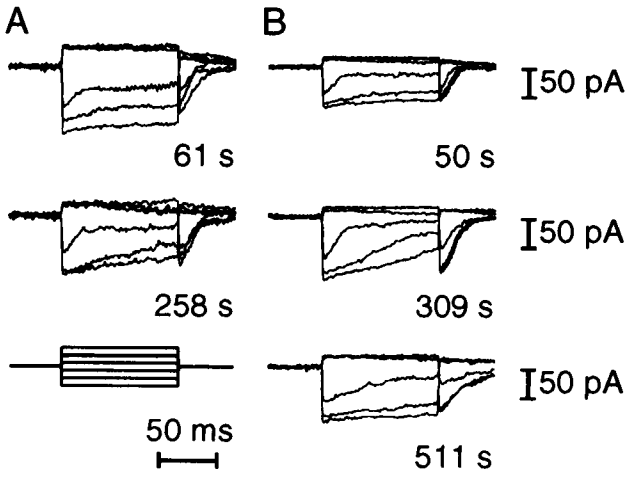

FIGURE 2 Measurement of adaptation by the mechanoelectrical-transduction process of hair cells in the presence of NEM. The transduction currents resulting from 100-ms hair-bundle deflections of ±100 nm, ±300 nm, and ±500 nm (*bottom traces* in *A*) were recorded at the times after the onset of whole-cell recording indicated under each set of traces. The inward transduction current, shown as a downward deflection, declined after the onset of stimulation as a result of adaptation to positive stimuli. Adaptation to negative stimulation was signaled by the rebound responses at the ends of the stimuli. (*A*) When dialyzed with a control solution lacking NEM, a hair cell exhibited robust adaptation for >4 min. The initial family of responses (*top traces*) was acquired as a control at the outset of recording; the lower family (*center traces*) was obtained after several minutes' recording. (*B*) When dialyzed with an internal solution containing 300 μM NEM, a hair cell displayed robust adaptation at the outset of recording (*top traces*); adaptation persisted for >8 min (*center* and *bottom traces*). The slowed relaxation of the transduction current toward the baseline following positive stimulation may have reflected dilatory Ca^{2+} clearance from stereocilia as a result of NEM's inhibition of Ca^{2+} pumps (Dumont et al., 1996). Each trace represents the averaged reponses to five bundle displacements; the calibration bars apply to both panels.

31%. By contrast, this treatment reduced labeling of the 160-kDa protein by 55 ± 24% and of the 230-kDa isozyme by 46 ± 21%.

We also ascertained that 300 μM NEM does not significantly inhibit the ATPase activity of recombinant amphibian myosin Iβ. Two myosin preparations retained an average of 99 ± 34% of their ATPase activities after NEM treatment. Like the photolabeling result, this observation probably ensues from the absence of cysteinyl residues in the portion of the active site comprising residues 613 through 623 of amphibian myosin Iβ (Metcalf et al., 1994; Solc et al., 1994), the isozyme proposed to mediate adaptation.

The other methods of estimating the actin-activated Mg^{2+}-ATPase activity of the myosin in hair bundles relied upon measurements of the activity *lost* when bundle preparations were treated with reagents expected to interfere with the adaptation motor's activity. Because there are no highly specific inhibitors of myosin, each of the three reagents was selected on the basis of its ability to occlude all or part of the binding interface of myosin and actin.

The peptide My1 replicates a segment of myosin Iβ that, by analogy to the crystal structure of the S1 fragment of chicken myosin II (Rayment et al., 1993), should be situated on the surface of the hydrophobic actin-binding site. In a control experiment, this peptide reduced the ATPase activity of skeletal-muscle myosin II in a concentration-dependent fashion, with 50% inhibition at a concentration of 100 μM (data not shown). A scrambled peptide with the same amino-acid composition as My1 had a negligible effect on the ATPase activity of skeletal-muscle myosin II.

When the peptide My1 was applied to bundle preparations at a concentration of 200 μM, the ATPase activity declined by 5.0 fmol × s^{-1} × SE^{-1}. This difference presumably reflected the activities of all myosin isozymes in hair bundles. After NEM treatment of samples, My1 reduced the ATPase activity of hair bundles by 1.8 ± 1.0 fmol × s^{-1} × SE^{-1} (n = 3), the contribution of NEM-insensitive myosin isozymes. The scrambled version of My1 did not alter the ATPase activity of isolated hair bundles.

A second set of experiments was performed with another peptide, Ac1. This molecule represents actin's highly conserved amino terminus, which constitutes one of myosin's binding sites (DasGupta and Reisler, 1991; Kögler et al., 1991; Johara et al., 1993). At a concentration of 830 μM, Ac1 diminished the NEM-insensitive ATPase activity of hair bundles by 1.6 ± 0.4 fmol × s^{-1} × SE^{-1} (n = 4).

The final inhibitor was S1-NEM, the *N*-ethylmaleimide-treated subfragment 1 of myosin II, which binds irreversibly to actin filaments, lacks ATPase activity (Pemrick and Weber, 1976), and arrests myosin-based motility (Lin et al., 1996). Treatment of hair-bundle preparations with 17 μM

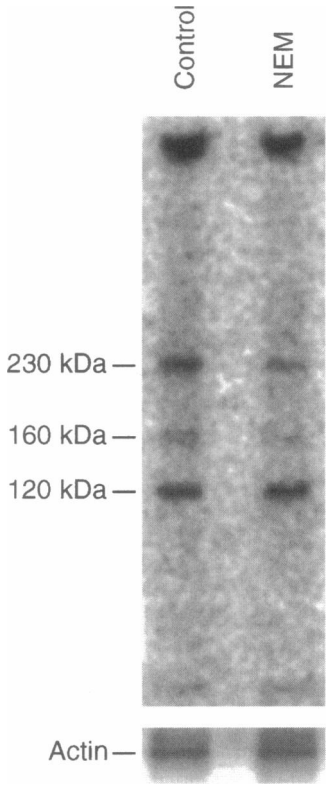

FIGURE 3 Effect of NEM on the photoaffinity labeling of hair-bundle myosin isozymes. Myosin isozymes from isolated hair bundles were photolabeled with vanadate-trapped [α-^{32}P]UDP and separated by SDS-PAGE; labeling was detected with a phosphor storage screen. The hair bundles analyzed in the right lane were incubated with 300 μM NEM before photolabeling, a step omitted for the control preparation. NEM had little effect on the nucleotide-hydrolytic activity of the 120-kDa myosin enzyme. By contrast, the activities of the 160-kDa and 230-kDa isozymes were significantly diminished by the sulfhydryl reagent. For each lane, the correponding silver-stained actin band provided an index of the amount of hair-bundle protein loaded.

S1-NEM reduced the NEM-insensitive ATPase activity by 2.4 $\pm$ 0.9 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 8).

It is conceivable that My1, Ac1, and S1-NEM affected different hair-bundle ATPases. Addition of either peptide after S1-NEM treatment, however, did not further diminish the ATPase activity (data not shown). We therefore conclude that My1, Ac1, and S1-NEM inhibited the same component of hair-bundle Mg^{2+}-ATPase activity, which we assume to have represented the actin-dependent activity of myosin.

Hair bundles might, in principle, contain ATPases in addition to myosin isozymes and the other constituents discussed earlier. We found, however, that addition of S1-NEM to bundles pretreated with thapsigargin, ouabain, and NEM resulted in essentially complete abolition of ATPase activity: the residual activity was only 0.7 $\pm$ 0.5 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 7).

A unique characteristic of some myosin isozymes is the activation of their ATPase activities by high concentrations of K^+ in the presence of EDTA. For hair-bundle myosin sensitive to competition by S1-NEM, however, our assay measured a K^+-EDTA ATPase activity of only 0.4 $\pm$ 0.2 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 27).

Effects of Ca^{2+}, calmidazolium, and calmodulin on myosin's ATPase activity

Ca^{2+} regulated the actin-dependent ATPase activity assayed after treatment of hair bundles with thapsigargin, ouabain, and NEM (Fig. 4). When the free Ca^{2+} concentration in the ATPase assay solution was decreased from the standard value of 10 μM to ~1 nM, the actin-dependent ATPase activity fell from 2.3 $\pm$ 0.4 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 8) to 1.0 $\pm$ 0.6 fmol $\times$ s^{-1} $\times$ SE^{-1} (n = 5). These two values were significantly different by a two-tailed, nonpaired t-test ($p < 0.02$). Raising the Ca^{2+} concentration above 10 μM did not produce a corresponding increase in ATPase activity; the average activity remained roughly constant, while the standard deviation increased.

The calmodulin inhibitor calmidazolium, which arrests adaptation and enhances photolabeling of myosin Iβ in hair bundles (Walker and Hudspeth, 1996), evidently interferes with the ATPase cycle of the adaptation motor. At a concentration of 5 μM, calmidazolium decreased the total ATPase activity of hair bundles by 4.6 fmol $\times$ s^{-1} $\times$ SE^{-1}. Added after such a calmidazolium treatment, the peptide Ac1 caused no further reduction of activity. In a separate experiment, S1-NEM decreased the ATPase activity by 2.2 fmol $\times$ s^{-1} $\times$ SE^{-1} in a control sample containing DMSO at the same concentration as that in the calmidazolium solution, but by only 0.7 fmol $\times$ s^{-1} $\times$ SE^{-1} in the presence of calmidazolium. We conclude that calmidazolium inhibits much of the myosin ATPase activity in hair bundles.

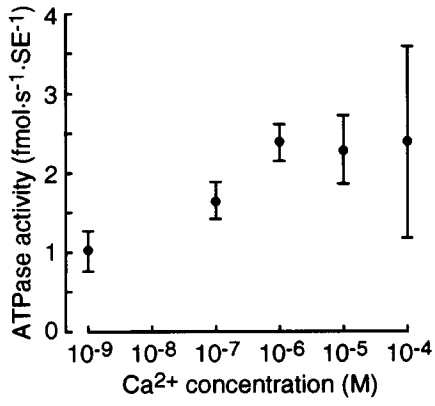

FIGURE 4 Effect of Ca^{2+} on the myosin ATPase activity of hair bundles. This response was measured as the component of hair-bundle ATPase activity inhibited by S1-NEM after pretreatment with thapsigargin, ouabain, and NEM. A data point represents four to seven measurements, each based upon a sample containing three saccular equivalents of hair bundles. EGTA was used to buffer the concentrations of free Ca^{2+} to the levels indicated.

Addition of calmodulin at concentrations as great as 100 μM did not affect the ATPase activity of hair-bundle myosin (data not shown). The presence of excess calmodulin, however, overcame calmidazolium's inhibitory effect on this activity.

DISCUSSION

ATPase activity assay

The development of in vitro molecular-motility assays and the recognition of the diversity of myosin isozymes have generated a need for highly sensitive methods to detect and quantitate the ATP consumption of these molecular motors. Visualization of the ATPase cycle of single myosin filaments (Sowerby et al., 1993), and even of individual myosin molecules (Funatsu et al., 1995), has been made possible by the use of fluorescent ATP analogs that can be observed while bound to purified myosin molecules. Impressive as they are, assays in this class cannot yet monitor the increased activity of myosin heads during their interaction with actin.

Colorimetric methods are generally sensitive to a few micromoles of inorganic phosphate; even the most sensitive reagents, such as malachite green, cannot detect less than nanomoles of phosphate (Ohno and Kodama, 1991). The use of radioactive ATP as substrate, followed by isolation of inorganic phosphate either on activated charcoal or by organic phase separation, can lower the detection limit to picomoles (Korn et al., 1982). The assay described here, a variant of this approach, took advantage of our ability to keep enzymes immobilized in the agarose gel used for bundle isolation. This feature enabled us to use reaction volumes of ≤1 μl, which proved sufficient to obtain separation by TLC and detection by scanning phosphor storage screens exposed to the TLC plates. By changing immersion solutions, we could assay the activity of the same set of bundles successively under different conditions; this procedure provided convenient internal controls. Under our assay conditions, the minimal detectable release of phosphate was approximately 0.1 fmol per sample.

Stereociliary myosin ATPases

Our assay measured the combined ATPase activities, not only of hair-bundle myosin isozymes, but also of several enzymes residing in hair bundles and perhaps of contaminants from hair-cell somata. The photolabeling results of Fig. 3 indicated, however, that our preparations contained little contamination (cf. Fig. 2 of Gillespie et al., 1993). Moreover, by the use of specific pharmacological inhibitors, we could measure the contributions of known ATPases and eliminate them from our determinations of myosin's ATPase activity. For example, thapsigargin, an inhibitor of Ca^{2+} pumps, induced the loss of 10–20% of the total ATPase activity in our samples. Ouabain occasionally inhibited some of the activity, a component possibly due to Na^+ pumps from the basolateral plasma membrane (Burnham and Stirling, 1984). A 160-kDa myosin isozyme, whose activity was partially inhibited by NEM, may have been myosin VI, which possesses a cysteinyl residue at its SH2 site (positions 686 and 694 for, respectively, *Drosophila melanogaster* and *Mus musculus*; Kellerman and Miller, 1992; Avraham et al., 1995). A second NEM-sensitive isozyme, of molecular mass 230 kDa, may have been myosin VIIa, which has a cysteinyl residue near the SH2 site (position 652 for *Homo sapiens*; Hasson et al., 1995). This signal may also have included some contribution from myosin II, a possible contaminant from the cell body (Drenckhahn et al., 1982; Gillespie et al., 1993).

The enzymatic activity of hair-bundle myosin isozymes in the presence of Ca^{2+} was estimated by inhibiting their interaction with actin and measuring the consequent loss of ATPase activity. The average actin-activated Mg^{2+}-ATPase activity of NEM-insensitive myosin molecules was about 2 fmol $\times$ s^{-1} per saccular equivalent of tissue, which comprised ~2500 hair bundles with ~50 stereocilia apiece. On the assumption that the rate of photolabeling by UTP's hydrolysis products (Fig. 3) mirrors the ATPase activity of hair-bundle myosin isozymes, ~50% of this activity may be attributed to myosin Iβ. If the number of myosin Iβ molecules in a single stereocilium is 100–200, as estimated on the basis of quantitative immunoblotting (Gillespie et al., 1993) and calmodulin binding (Walker and Hudspeth, 1996), then the specific Mg^{2+}-ATPase activity of a myosin Iβ molecule is 25–50 s^{-1}. Although this value substantially exceeds those previously reported for myosin I (Barylko et al., 1992; Zot et al., 1992; Wolenski et al., 1993; Zhu et al., 1996), it resembles the activity of myosin II when extrapolated to infinite actin concentration (Ferenczi et al., 1995) or after cross-linking to actin filaments (Mornet et al., 1981). The high ATPase activity that we observed may therefore reflect the great concentration of actin microfilaments in the stereociliary cytoskeleton and possibly the close apposition to these microfilaments of the myosin molecules thought to be congregated at insertional plaques (Gillespie et al., 1993; reviewed in Hudspeth and Gillespie, 1994). It should also be noted that our assays were conducted on myosin molecules in situ and usually only hours post mortem; under these circumstances, myosin might display an ATPase activity substantially greater than that following extensive and protracted purification procedures.

When hair cells were dialyzed with NEM, both mechanoelectrical transduction and adaptation persisted for the duration of whole-cell recording. Although we cannot rule out the possibility that NEM failed to reach the site of adaptation in these recordings, other small molecules are effective at inhibiting adaptation within shorter periods of intracellular dialysis. ADP and its analog ADPβS (Gillespie and Hudspeth, 1993), as well as the phosphate-ion analogs vanadate and beryllium fluoride (Yamoah and Gillespie, 1996), arrest adaptation by interrupting the motor molecules' ATPase cycle; calmidazolium and peptide IV interfere with the participation of calmodulin in adaptation

(Walker and Hudspeth, 1996). We therefore conclude that NEM at the concentration used does not significantly affect the adaptation machinery in situ, and hence that the motor molecules involved are not highly sensitive to this sulfhydryl reagent.

By three criteria, the actin-dependent ATPase that we believe to be myosin Iβ behaves as expected of the mechanoenzyme mediating adaptation. First, this isozyme and the motor molecule share a low sensitivity to NEM. Second, as anticipated from the role of Ca^{2+} in regulating adaptation (Eatock et al., 1987; Assad et al., 1989; Hacohen et al., 1989), this enzyme's activity nearly doubles when the Ca^{2+} concentration rises from a negligible value into the micromolar range in which calmodulin is activated (reviewed in Cox et al., 1988). The entry of Ca^{2+} into stereocilia might therefore reduce the tension in gating springs by altering the fraction of the ATPase cycle that each myosin molecule spends in force-producing states (reviewed in Hudspeth and Gillespie, 1994) or by dislodging calmodulin light chains and thus arresting activity altogether (Zhu et al., 1996). Finally, consistent with adaptation's arrest by calmodulin inhibitors (Walker and Hudspeth, 1996), calmidazolium significantly inhibits this ATPase. Although the proof that a particular myosin isozyme effects adaptation awaits the development of an isozyme-specific probe such as a blocking antibody, the present results strengthen the evidence that myosin Iβ participates in adaptation.

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