Calmodulin controls adaptation of mechanoelectrical transduction by hair cells of the bullfrog’s sacculus

(auditory system/calmidazolium/hair bundle/myosin/vestibular system)

RICHARD G. WALKER* AND A. J. HUDSPETH

Howard Hughes Medical Institute and Laboratory of Sensory Neuroscience, The Rockefeller University, Box 314, 1230 York Avenue, New York, NY 10021-6399

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ABSTRACT Deflection of the mechanically sensitive hair bundle atop a hair cell opens transduction channels, some of which subsequently reclose during a Ca2+-dependent adaptation process. Myosin I in the hair bundle is thought to mediate this adaptation; in the bullfrog’s hair cell, the relevant isozyme may be the 119-kDa amphibian myosin Iβ. Because this molecule resembles other forms of myosin I, we hypothesized that calmodulin, a cytoplasmic receptor for Ca2+, regulates the ATPase activity of myosin. We identified an ~120-kDa calmodulin-binding protein that shares with hair-bundle myosin I the properties of being photolabeled by vanadate-trapped uridine nucleotides and immunoreactive with a monoclonal antibody raised against mammalian myosin Iβ. To investigate the possibility that calmodulin mediates Ca2+-dependent adaptation, we inhibited calmodulin action and measured the results with two distinct assays. Calmodulin antagonists increased photolabeling of hair-bundle myosin I by nucleotides. In addition, when introduced into hair cells through recording electrodes, calmodulin antagonists abolished adaptation to sustained mechanical stimuli. Our evidence indicates that calmodulin binds to and controls the activity of hair-bundle myosin I, the putative adaptation motor.

Hair cells line the auditory and vestibular receptor organs of vertebrates, where they transduce mechanical stimuli into electrical signals that are transmitted to the brain (reviewed in ref. 1). The organelle that responds to these mechanical stimuli, the hair bundle, comprises a few dozen to a few hundred actin-filled processes termed stereocilia. Forces applied to a hair bundle open mechanically sensitive cation channels, producing a transduction current carried by K+ and, to a lesser extent, Na+ and Ca2+. Channel gating is probably controlled by the tension in tip links that interconnect adjacent stereocilia.

The great mechanical sensitivity of the hair bundle confers a potential disadvantage: protracted stimulation might saturate the transducer. To avoid this problem, the hair cell adapts to deflections of its hair bundle (reviewed in ref. 2). At the hair bundle’s resting position, the transduction channels’ open probability is ~0.15. In response to a positive stimulus, additional channels open, increasing the flow of inward current. Over tens of milliseconds, however, the open probability declines until the current returns nearly to its original level. When the bundle is restored to its resting position, almost all the channels close, then some reopen with a similar time course. If the bundle is instead deflected in the negative direction, the channels’ open probability first decreases, then rebounds toward the initial value. When the bundle is restored to its resting position, the open probability increases sharply before returning to the resting level.

The prevailing model of adaptation supposes that a molecular motor at the upper end of each tip link regulates the tip-link tension by sliding down or climbing up the actin core of the stereocilium (2–4). This adaptation motor is thought to comprise a cluster of ~50 myosin molecules (2, 5), perhaps of the isozyme myosin Iβ (119 kDa; refs. 6 and 7), myosin VI (142 kDa; ref. 8), or myosin VIIa (~240 kDa; ref. 9). The rate of slipping or climbing by the adaptation motor is regulated by the cytoplasmic Ca2+ concentration (10, 11), which is determined in part by Ca2+ influx through transduction channels near the stereociliary tip (12).

Calmodulin, a cytoplasmic receptor for Ca2+ in many cellular processes, occurs in the hair bundle at an average concentration of ~70 μM (13, 14). Because calmodulin binds to and regulates various myosin isozymes (15–17), it is plausible that calmodulin mediates the effects of Ca2+ on the adaptation motor. To assess this possible role of calmodulin, we have used a calmodulin-alkaline phosphatase conjugate (CaM-AP; ref. 14) to seek calmodulin-binding myosin isoforms in isolated hair bundles. To correlate calmodulin activity with adaptation, we have additionally explored the effects of calmodulin inhibitors on the nucleotide-hydrolytic activity of hair-bundle myosin I and on physiologically measured adaptation.

MATERIALS AND METHODS

Hair-Bundle Isolation and Detergent Extraction. Hair bundles were isolated from the sacculi of bullfrogs (Rana catesbeiana) by the twist-off technique (18). Agarose blocks containing purified hair bundles were excised, combined to give similar numbers of bundles per sample, quickly frozen in liquid N2, and stored at -80°C. Membranes were disrupted by freezing and thawing in 22.5 μl of isolation solution, which contained 100 mM KCl, 2.5 mM MgCl2, 20 μM CaCl2, 2.5 mM 2-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin, 200 μM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; Boehringer Mannheim), and 25 mM Hepes, pH 7.5. After centrifugation at 29,000 × g for 15 min, the supernatant fluid was removed and retained for analysis. The insoluble material was extracted for 30 min with the same solution containing 1% (vol/vol) Triton X-100 and sedimented as above. Both the supernatant fluid and the sedimented material were saved for analysis.

CaM-AP Binding to Hair-Bundle Myosin I. Although the conjugate of calmodulin to alkaline phosphatase (CaM-AP) provides a sensitive probe for calmodulin-binding proteins, this reagent previously failed to detect myosin I in hair bundles (14). Subsequent experiments disclosed that purified mammalian myosin Iβ (MMIβ) was prevented from binding CaM-AP in a blot-overlay assay if casein was present in the blocking

Abbreviations: MMIβ, mammalian myosin Iβ; CaM-AP, calmodulin-alkaline phosphatase conjugate; DMSO, dimethyl sulfoxide.

*Present address: Departments of Neurosciences and Biology, University of California, San Diego, La Jolla, CA 92037-0649.
solution or if a nonionic detergent was absent. When gelatin was employed as a blocking reagent and Tween 20 (Fluka) was included, CaM-AP was capable of detecting as little as 200 pg (1.7 fmol) of MMIB. The interaction between CaM-AP and MMIB was Ca$^{2+}$ dependent, as 1 mM EGTA abolished binding.

Purified MMIB was subjected to SDS/PAGE through 7.5% acrylamide minigels. The gels were incubated with two, 10-min changes of a solution containing 0.25 g of hemoglobin per liter, 5% (vol/vol) methanol, and 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11. Proteins were electrophoretically transferred in 10 mM CAPS and 5% methanol to polyvinylidene difluoride membranes (PVDF; Millipore), which were then incubated for 1 h in a solution containing 150 mM NaCl and 25 mM sodium ethylenecitric acid (HEPES) buffered to pH 7.5 with 25 mM Hepes (Hepes-buffered saline solution, HBSS). Remaining protein-binding sites on the membranes were saturated for 2–4 h with a blocking solution of 9% (wt/vol) cold-water fish gelatin/0.5% Tween 20/100 M CaCl$_2$ in HBSS. After blocking was complete, membranes were incubated for 1–2 h in a similar solution containing CaM-AP at a final concentration of 1.2 mg/liter. CaM-AP was detected on PVDF membranes after washing with three, 5-min changes of 0.3% Tween 20/0.1 M CaCl$_2$/1 M MgCl$_2$/50 M 2-aminoo-2-methyl-1-propanol, pH 10.3. Charged nylon membranes were incubated for 5 min in the latter solution containing 400 M chemiluminescence substrate disodium 3-(4-methoxy-spiro[1,2-dioxetane-3,2'-5'-chloro]tricyclo[3.3.1.1$^{3,7}$decane]-4)-phylin phosphatase (CSPD) and 0.5 g of chemiluminescence enhancer (Nitro-Block, Tropix, Bedford, MA) per liter, then treated as described (18).

To examine the subcellular localization and electrophoretic mobility of photolabeled proteins, calmodulin-binding proteins, and immunologically related forms of myosin I, we photolabeled bundles by a modification of a published procedure (5). Isolated bundles were permeabilized with 80 mg of saponin per liter in isolation solution, exposed to radioactively labeled nucleotide for 40 min in the presence of vanadate ion (VO$_4^{3-}$) and 20 M Ca$^{2+}$, and photolabeled as detailed below. After these hair bundles were combined with unlabeled bundles and extracted as described above, the fractions were placed in SDS/PAGE sample solution [65 mM Tris-HCl, pH 6.8/5 M EDTA/1.5% (wt/vol) SDS/350 M 2-mercaptoethanol/0.01% bromophenol blue/680 mM glycerol].

After SDS/PAGE, transfer, and incubation in HBSS, the moistened membranes were wrapped in plastic and exposed for 24 h to a phosphor imaging plate. The amounts of radioactivity corresponding to the phosphor signals (Phosphor-Imager, Molecular Dynamics) were determined with image-analysis software (IMAGEQUANT, version 3.3, Molecular Dynamics; IPLABLEGEL, version 1.5c, Signal Analytics, Vienna, VA). To eliminate the possibility that radioactivity on a blot accounted for any signal observed during the subsequent steps, the membranes were exposed to x-ray film for 1 h; no signal was observed on these control exposures.

Membranes were rewetted in HBSS, blocked, and incubated with CaM-AP. After the conjugate had been detected as described above, the residual CaM-AP and substrate were stripped from the membranes by four, 10-min washes with 1% SDS/1 mM EGTA. The blots were blocked again for 2 h with a casein-containing solution (18). To ensure that stripping had removed all bound phosphatase activity, the membranes were incubated in chemiluminescence substrate and enhancer and exposed to film. If any chemiluminescence signal remained, the blots were stripped again until no signal was observed. Membranes were then incubated in casein-containing blocking solution for 1 h and probed for 2 h with 10 mg of the anti-MMIB monoclonal antibody mT2 per liter (19); bound antibody was detected as described (5).

**RESULTS**

**Hair Bundles Contain an ~120-kDa Calmodulin-Binding Protein.** To test for calmodulin binding to myosin I in hair bundles, isolated bundles were extracted and the proteins were transferred to a membrane. When this blot was probed with CaM-AP, a protein of molecular mass ~120 kDa was observed in the detergent-insoluble subcellular fractions (Fig. 1A). Like
the other constituents of hair bundles, this protein did not bind CaM-AP in the presence of 1 mM EGTA (Fig. 1B). Assuming that the ~120-kDa calmodulin-binding protein represents myosin I and using purified MM1β as a standard, we conducted quantitative CaM-AP blot-overlay assays to estimate that the combined hair bundles of one saccus contain 5 ± 2 pg (n = 3) of myosin I.

**CaM-AP Binds to Hair-Bundle Myosin I.** To test whether the calmodulin-binding molecule demonstrated by the CaM-AP overlay technique might be hair-bundle myosin I, we correlated CaM-AP binding with two properties of this isozyme (5): susceptibility to photocrosslinking with radiolabeled nucleotides and immunoreactivity toward a monoclonal antibody, mT2, raised against MM1β.

Incubation of hair bundles with [α-32P]UTP, vanadate trapping of hydrolysis products, and exposure to ultraviolet light led to photolabeling of proteins with approximate molecular masses of 120, 160, and 230 kDa (Fig. 1C). Densitometry of the ~120-kDa band indicated that 82% ± 12% (n = 3) of this protein was resistant to detergent extraction. The remainder was found principally in the detergent-soluble fraction, while <1% was soluble in the buffered solution alone. To identify calmodulin-binding proteins, the same membrane was stripped and probed with CaM-AP. The labeled proteins included an ~120-kDa protein in the insoluble fraction (Fig. 1D). Finally, to determine on the same blot the migration and distribution of hair-bundle myosin I, we employed the monoclonal antibody mT2 (5, 19). This antibody recognized an ~120-kDa protein that was resistant to detergent extraction (Fig. 1E). The proportion of ~120-kDa protein present in the detergent-insoluble fractions was 79% ± 9% (n = 3); the remainder of the myosin occurred in the detergent-soluble fraction.

**Calmodulin Inhibitors Increase Photolabeling of an ~120-kDa Protein.** The ability of hair-bundle myosin I to be photocrosslinked to radiolabeled nucleotides reflects the nucleotide-hydrolytic activity of the isozyme (5). To test whether calmodulin regulates the activity of hair-bundle myosin I, we tested the effect of calmodulin inhibitors on photolabeling of the ~120-kDa protein.

When permeabilized hair bundles were treated with calmodulin inhibitors, the incorporation of radiolabel doubled;

**Fig. 1.** Myosin I in hair bundles exhibits Ca2+-dependent binding of calmodulin. (A and B) Preparations of ~200,000 isolated hair bundles each were extracted sequentially with buffer solution (S) and with a similar solution containing Triton X-100 (T). The two supernatant fluids, as well as the final pellet (P), were analyzed on blots. CaM-AP detected an ~120-kDa protein in a solution containing 100 μM Ca2+ (A) but not in the presence of 1 mM EGTA (B). (C-E) In each preparation, ~42,000 isolated hair bundles were photolabeled with [α-32P]UTP and combined with an additional ~220,000 bundles. After bundles had been extracted with a buffer solution alone (S) or one containing Triton X-100 (T), the supernatants and insoluble material (P) were subjected to SDS/PAGE and transferred to a membrane. (C) Radioactivity due to photolabeling was determined by exposure to a phosphor storage plate. The nucleotide labeled ~120-, ~160-, and ~230-kDa proteins, which were predominantly insoluble. (D) CaM-AP identified an ~120-kDa protein that migrated with a mobility identical to that of the photolabeled protein. (E) Monoclonal antibody mT2 recognized hair-bundle myosin I as an ~120-kDa protein. A dark bar to the right of each panel marks the position of 120-kDa protein.

**Fig. 2.** Calmodulin inhibitors enhance photolabeling of hair-bundle myosin I. (A) Calmidazolium increases the photolabeling of an ~120-kDa hair-bundle protein. Four samples, each containing ~13,500 isolated hair bundles, were treated for 15 min with permeabilization solution, calmidazolium with DMSO, calmidazolium followed by calmodulin, or DMSO alone. The control designation refers to the presence of DMSO in a sample. (B) Peptide IV also augments photolabeling of an ~120-kDa protein. Four samples, each containing ~25,000 bundles, were treated for 15 min with permeabilization solution, peptide IV, peptide IV followed by calmodulin, or scrambled peptide IV. The control designation refers to the presence in one sample of scrambled peptide IV. The amount of radioactivity in the ~120-kDa band of each lane, normalized to the density of the corresponding actin band (bottom panels), is expressed immediately above each lane as a percentage of the value for the sample exposed to permeabilization solution alone. The molecular-mass indicators in B correspond to those in A; the dark bar at the right of each panel marks the position of 120-kDa protein.
for both calmidazolium ($n = 4$) and peptide IV ($n = 5$), labeling was $210\% \pm 80\%$ that of control samples. As negative controls for the inhibitors, the DMSO solvent or a scrambled version of peptide IV changed the amount of photolabeling negligibly, to respectively $90\% \pm 13\% (n = 4)$ or $105\% \pm 31\% (n = 5)$ that of controls. As a test of the reversibility of the inhibitors' effects, bovine brain calmodulin was added to samples that had previously been treated with calmidazolium or peptide IV; the extent of photolabeling then returned to respectively $86\% \pm 21\% (n = 3)$ or $90\% \pm 13\% (n = 5)$ of control levels. Treatment of samples with calmodulin alone had no effect on photolabeling.

**Calmidazolium Inhibitors Abolish Adaptation.** Using whole-cell, tight-seal electrodes, we recorded mechanically activated currents from isolated hair cells. Adaptation manifested itself as a decline in the transduction current during a positive stimulus and as an inward current transient following a negative stimulus (Fig. 3A). When control internal solutions were used in recording electrodes, robust adaptation persisted for $>10$ min in seven of nine cells.

When calmidazolium was included in electrode solutions, a hair cell demonstrated clear adaptation to both positive and negative stimuli shortly after rupture of its membrane. Over the next $\sim 7$ min, however, the rate and extent of adaptation gradually declined until adaptation was almost abolished (Fig. 3B). The same effect was observed in 8 of 10 cells that met the criteria for inclusion in the data set. In addition to blocking adaptation, calmidazolium reduced the magnitude of the transduction current; in the example shown, the response diminished from $-365$ pA to $-160$ pA. Decrements in transduction current were observed in both control and experimental cells, but the magnitude of the decrease was generally greater in cells treated with inhibitors.

We also tested the effect of peptide IV on mechanoelectrical transduction. Within several minutes, this calmodulin inhibitor abolished adaptation in seven of eight cells (Fig. 3C). The peak transduction current concomitantly declined to about half its initial amplitude. For cells dialyzed against either inhibitor, displacement-response relations indicated that no significant change occurred in the transduction channels' open probability at the hair bundles' resting positions (data not shown).

To eliminate the possibility that the solvating agent for calmidazolium affected adaptation, we included DMSO in control electrode solutions at a concentration identical to that in the calmidazolium experiments. Transduction and adaptation were virtually unchanged during several minutes of recording from all four cells examined (Fig. 3D). As a second control, scrambled peptide IV was also tested in whole-cell recording electrodes; a normal response then endured through several minutes of recording from five of six cells (Fig. 3E).

**DISCUSSION**

**Hair-Bundle Myosin I Binds Calmodulin.** Hair bundles from the bullfrog's saccus contain a calmodulin-binding protein whose abundance, molecular mass, and susceptibility to detergent extraction correspond to those of myosin I (5). On the basis of quantitative overlay experiments and in agreement with the earlier study, myosin I occurs at $\sim 10,000$ molecules per hair bundle, or nearly 200 molecules per stereocilium.

Because $Ca^{2+}$ controls the nucleotide photolabeling of hair-bundle myosin I, the ion presumably affects a step in the ATPase cycle of the isozyme (5). Many myosin I isozymes exhibit $Ca^{2+}$-dependent ATPase activity that is regulated by calmodulin light chains (15-17). Because hair-bundle myosin I binds calmodulin in a $Ca^{2+}$-dependent manner, calmodulin may confer upon this isozyme the ability to measure the local $Ca^{2+}$ concentration and thereby to regulate the adaptation motor.

**Calmodulin Controls Adaptation.** To confirm that calmodulin mediates the effects of $Ca^{2+}$ on adaptation, we dialyzed cells against inhibitors of calmodulin binding and observed slowing and eventual blockage of adaptation to sustained displacements. A preliminary report (22) noted comparable phenomena following application of several inhibitors during extracellular recordings of electrical responses. That chemically distinct antagonists produced similar effects suggests these inhibitors specifically affected a calmodulin-mediated process.

For calmidazolium and peptide inhibitors to be effective, calmodulin must be free from its targets (20). We observed a delay of several minutes between gaining access to a cell and complete blockage of adaptation. Because $Ca^{2+}$ continuously permeates the transduction channels in an undisturbed hair bundle (12), $Ca^{2+}$ may be present in stereocilia at micromolar concentrations. In the presence of $Ca^{2+}$, about half the calmodulin in stereocilia is soluble (14); we therefore expect that free calmodulin exchanges with that bound to its receptors. Our results suggest that myosin I molecules slowly release calmodulin light chains into the stereociliary cytoplasm, where they are promptly sequestered by inhibitors. Over time this strips the myosin molecules of their light chains and thereby reduces responsiveness to changes in $Ca^{2+}$ concentration.

**Fig. 3.** Calmodulin inhibitors abolish adaptation. Mechanoelectrical transduction currents were recorded while hair bundles were subjected to 100-nm stimuli of $\pm 100$ nm, $\pm 300$ nm, and $\pm 500$ nm. (A) During recordings with an electrode that contained no inhibitors, transduction and adaptation were robust after rupture of the cell plasma membrane (upper traces) and remained so for $\sim 8$ min (lower traces). (B) When $4 \mu M$ calmidazolium was included in the electrode, a cell exhibited brisk adaptation shortly after electrical access was obtained (upper traces); after $\sim 7$ min, however, the transduction current diminished and adaptation was abolished (lower traces). (C) Inclusion in the recording electrode of $500 \mu M$ peptide IV produced no noticeable effects on transduction shortly after onset of whole-cell recording (upper traces), but adaptation disappeared within $\sim 7$ min (lower traces). No effect was observed when a control substance, $70 \mu M$ DMSO (D) or $500 \mu M$ scrambled peptide IV (E), was placed in the recording electrode. A family of traces represents the average of six responses to each stimulus amplitude. The time at the lower right of each family indicates the interval between rupture of the cell membrane and onset of data collection. The calibration bars apply to all traces.
With one important difference, the effects of the calmodulin inhibitors resembled those of other treatments that abolish adaptation. Unlike lowering the stereocilia Ca\(^{2+}\) concentration (10, 11) or dialysis against adenine nucleotide analogs (21), treatment with calmodulin inhibitors did not increase the resting open probability of the transduction channels. The inhibited adaptation motors therefore remained attached to the cytoskeleton but apparently did not produce enhanced force on the gating springs. There is precedent for such a response, for a calmodulin inhibitor arrests the motion of actin filaments along a myosin V-coated coverslip, leaving the filaments tightly bound (23). In hair cells, removal of calmodulin from myosin molecules may similarly halt movement of an adaptation motor along the actin cytoskeleton and freeze the motor there.

**Calmodulin Controls the Nucleotide-Hydrolytic Activity of Hair-Bundle Myosin I.** Measurement of the relative amount of nucleotide incorporated in preparations of permeabilized hair bundles provided an indirect assay of the nucleotide-hydrolytic activity of hair-bundle myosin I. Calmodulin inhibitors increased the amount of radiolabeled nucleotide crosslinked to the \(\approx 120\)-kDa hair-bundle protein, probably myosin I\(\beta\). Although not quantitated here, labeling was also enhanced for the \(\approx 160\)-kDa and \(\approx 230\)-kDa proteins, which may be myosin VI (8) and myosin VIIa, respectively (9). Increased radiolabeling of hair-bundle myosin I was not observed if inhibitor treatment was followed by addition of exogenous calmodulin, indicating that the effects seen were reversible and specific for a calmodulin-mediated process.

It is possible to explain both the physiological and the photolabeling results in terms of the myosin ATPase cycle. In the first step of this cycle, a myosin molecule binds ATP and dissociates from its rigor complex with actin. Hydrolysis of the nucleotide triggers reassociation and release of inorganic phosphate ion; the resultant state is that in which vanadate ion can trap nucleoside diphosphate at the active site. Following the power stroke and a kinetically irreversible step, myosin finally releases ADP and resumes the rigor state. If calmodulin must be present for the power stroke and kinetically irreversible step to occur, calmodulin inhibitors would trap adaptation-motor molecules in a tight-binding state antecedent to the power stroke, leaving the open probability of transduction channels near its resting level. At the same time, increasing the occupancy of the actin–myosin–ADP state would enhance vanadate trapping and subsequent photolabeling. It is thought that the adaptation motor slips down or ascends a stereocilium, depending upon the fraction of the ATPase cycle spent in respectively the weak- or the tight-binding state (reviewed in ref. 2). Because raising the Ca\(^{2+}\) concentration promotes slipping adaptation and decreases the affinity of the \(\approx 120\)-kDa protein for ADP (5), Ca\(^{2+}\) binding by calmodulin may regulate adaptation by accelerating ADP release from myosin I\(\beta\).

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