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

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

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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 myo- 

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 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 isoforms (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 A, 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 solution.

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

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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 MMIβ. The interaction between CaM-AP and MMIβ was Ca²⁺ dependent, as 1 mM EGTA abolished binding.

Purified MMIβ 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 ethylmercurithiosalicylate 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 μM CaCl₂ in HBSS. After blocking was complete, membranes were incubated for 1–2 h in a similar solution containing CaM-AP at a 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 mM CaCl₂/1 mM MgCl₂/50 mM 2-amino-2-methyl-1-propanol, pH 10.3. Charged nylon membranes were incubated for 5 min in the latter solution containing 400 μM chemiluminescence substrate disodium 3-(4-methoxy-spiro[1,2-dioxetane-3,2′:5′-chboro)tricyclo[3.3.1.1³,7]decan]-4-yl)-phenyl phosphate (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 solution containing MnCl₂ rather than MgCl₂. Bundles were next exposed for 15 min to permeabilization solution alone or permeabilization solution containing 4 μM calmidazolium chloride, 70 μM DMSO, or 70 μM DMSO and either 500 nM peptide IV or 500 nM scrambled peptide IV. After the reagents had been washed away by three, 5-min exchanges of the permeabilization solution, bundles were treated with permeabilization solution with or without 10 μM bovine brain calmodulin (Calbiochem, La Jolla, CA). Hair bundles were incubated with 1–2 μM [α-32P]UTP (~10¹⁷ Bq/mol; Amersham Corp., Arlington Heights, IL) in a reaction mixture consisting of permeabilization solution to which UMP and vanadate ion were each added at a concentration of 1 mM. The bundles were exposed to this solution for 15 min, approximately half the time required for maximal labeling.

After having been washed for 60 min with six changes of permeabilization solution, bundles were irradiated for 20 min at 0°C with 254-nm light. Proteins were separated by SDS/PAGE on 4–15% gradient minigels, which were then fixed and stained with silver nitrate. Dried gels were exposed to phosphor storage plates for 12–48 h. Because the lanes could not be loaded identically, radiolabeling was normalized to the content of actin (5), the dominant protein of hair bundles (18), in each lane. Each numerical result is presented as the mean ± SD for n experiments.

Electrophysiological Recording. The recording environment has been described (21). Solitary hair cells, isolated from saccular epithelia of bullfrogs by published methods (12), were allowed to settle onto the concanavalin A-coated bottom of the recording chamber. Maintained at ~20°C in oxygenated standard saline solution (12), cells were viewed with an inverted microscope (IM-35; Zeiss) equipped with a ×63 objective lens and differential-interference-contrast optics. Transduction currents were recorded by the tight-seal, whole-cell method through electrodes containing 110 mM Cs⁺, 2 mM Na⁺, 3 mM Mg²⁺, 106 mM Cl⁻, 2 mM ATP, 1 mM EGTA, and 5 mM Hepes, pH 7.25. After correction for the +4-mV junction potential of the electrode solutions, the membrane potential of each cell was clamped at ~70 mV. The axial resistance of a recording electrode was 2–3 MΩ in the bath solution but usually increased to 10–20 MΩ during recording. When the access resistance exceeded 20 MΩ, gentle suction was applied through the electrode; if no improvement in the electrical access was observed, the data from the cell were discarded. Forty successful experiments were performed, in which maximal transduction currents ranged from ~100 pA to ~360 pA.

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 Calmodulin Inhibitors. Calmidazolium chloride (Calbiochem-Novabiochem) was dissolved in dimethyl sulfoxide (DMSO) before use. A custom synthesis produced peptide IV (LKWWKLLKWLKLKLG), a positively charged, amphiphilic, α-helical molecule with a dissociation constant for calmodulin binding of 210 pM (20). A second synthesis yielded scrambled peptide IV (LKLLKWLKLKLG), which contains identical amino acid residues but is expected neither to form an α-helix nor to bind calmodulin.

Vanadate Trapping and Photoaffinity Labeling. To provide an estimate of the nucleotide-hydrolytic activity of the ~120-kDa hair-bundle myosin I, vanadate trapping and photolabeling of hair bundles were performed essentially as described (5). Isolated, agarose-embedded hair bundles were permeabilized for 10 min with 80 mg of saponin per liter in isolation solution containing MnCl₂ rather than MgCl₂. Bundles were next exposed for 15 min to permeabilization solution alone or permeabilization solution containing 4 μM calmidazolium chloride, 70 μM DMSO, or 70 μM DMSO and either 500 nM peptide IV or 500 nM scrambled peptide IV. After the reagents had been washed away by three, 5-min exchanges of the permeabilization solution, bundles were treated with permeabilization solution with or without 10 μM bovine brain calmodulin (Calbiochem, La Jolla, CA). Hair bundles were incubated with 1–2 μM [α-32P]UTP (~10¹⁷ Bq/mol; Amersham Corp., Arlington Heights, IL) in a reaction mixture consisting of permeabilization solution to which UMP and vanadate ion were each added at a concentration of 1 mM. The bundles were exposed to this solution for 15 min, approximately half the time required for maximal labeling.

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Fig. 1. Myosin I in hair bundles exhibits Ca\textsuperscript{2+}-dependent binding of calmodulin. (A and B) Preparations of \(\approx 200,000\) 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 \(\sim 120\)-kDa protein in a solution containing 100 \(\mu\text{M}\) Ca\textsuperscript{2+} (A) but not in the presence of 1 \(\text{mM}\) EGTA (B). (C–E) In each preparation, \(\approx 42,000\) isolated hair bundles were photolabeled with \([\alpha-\text{32P}]\text{UTP}\) and combined with an additional \(\sim 220,000\) bundles. After bundles had been extracted with a buffered 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 \(\sim 120-, \sim 160-,\) and \(\sim 230\)-kDa proteins, which were predominantly insoluble. (D) CaM-AP identified an \(\sim 120\)-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 \(\sim 120\)-kDa protein. A dark bar to the right of each panel marks the position of 120-kDa protein.

When permeabilized hair bundles were treated with calmodulin inhibitors, the incorporation of radiolabel doubled;
for both calmidazolium (Fig. 2A; n = 4) and peptide IV (Fig. 2B; n = 5), labeling was 210% ± 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% ± 13% (n = 4) or 105% ± 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% ± 21% (n = 3) or 90% ± 13% (n = 5) of control levels. Treatment of samples with calmodulin alone had no effect on photolabeling.

**Calmodulin 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 ≈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 sacculus 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 ≈10,000 molecules per hair bundle, or nearly 200 molecules per stereocilium.

Because Ca2+ 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 isoforms exhibit Ca2+-dependent ATPase activity that is regulated by calmodulin light chains (15–17). Because hair-bundle myosin I binds calmodulin in a Ca2+-dependent manner, calmodulin may confer upon this isozyme the ability to measure the local Ca2+ concentration and thereby to regulate the adaptation motor.

**Calmodulin Controls Adaptation.** To confirm that calmodulin mediates the effects of Ca2+ 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 Ca2+ continuously permeates the transduction channels in an undisturbed hair bundle (12), Ca2+ may be present in stereocilia at micromolar concentrations. In the presence of Ca2+, 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 Ca2+ concentration.

![Graph](image)

**Fig. 3.** Calmodulin inhibitors abolish adaptation. Mechanoelectrical transduction currents were recorded while hair bundles were subjected to 100-ms stimuli of ±100 nm, ±300 nm, and ±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 ∼8 min (lower traces). (B) When 4 μM calmidazolium was included in the electrode, a cell exhibited brisk adaptation shortly after electrical access was obtained (upper traces); after ∼7 min, however, the transduction current diminished and adaptation was abolished (lower traces). (C) Inclusion in the recording electrode of 500 nM peptide IV produced no noticeable effects on transduction shortly after onset of whole-cell recording (upper traces), but adaptation disappeared within ∼7 min (lower traces). No effect was observed when a control substance, 70 μM DMSO (D) or 500 nM 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 stereociliary 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 ~120-kDa hair-bundle protein, probably myosin Iβ. Although not quantitated here, labeling was also enhanced for the ~160-kDa and ~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 antecedant 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 ~120-kDa protein for ADP (5), Ca^{2+} binding by calmodulin may regulate adaptation by accelerating ADP release from myosin Iβ.

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