Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 2807–2811, April 1993
Neurobiology

Calmodulin and calmodulin-binding proteins in hair bundles

(alkaline phosphatase/auditory system/chemiluminescence/hair cell/ligand blot)

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Contributed by A. J. Hudspeth, December 23, 1992

ABSTRACT **Calcium ion plays an important role in the hair cell's mechanoelectrical transduction process; in particular, Ca^{2+} controls adaptation to protracted mechanical stimuli. Because calmodulin is a ubiquitous intracellular receptor for Ca^{2+} and has been shown to accumulate at the tips of stereocilia, we determined its concentration and identified the proteins with which it interacts in the hair bundle. By performing quantitative immunoblot analysis on isolated bundles, we ascertained that the average concentration of calmodulin within each stereocilium is ≈70 μM. Extraction experiments disclosed that, in the presence of 20 μM Ca^{2+}, 50% of the calmodulin is bound to detergent-soluble receptors. To distinguish these receptors, we developed an assay that utilizes calmodulin crosslinked to alkaline phosphatase. This technique is ≈100-fold more sensitive than calmodulin-binding assays that employ ^{125}I- or biotin-labeled calmodulin. When used with chemiluminescence detection in a blot-overlay assay, the calmodulin–alkaline phosphatase conjugate identified hair-bundle proteins of molecular masses 25, 35, 145, 175, 240, and 350 kDa. We examined the subcellular distribution of these receptors; all but the 240-kDa molecule are soluble in a nonionic detergent. The relatively high concentration of calmodulin and the presence of several calmodulin-binding proteins provide evidence for a role of calmodulin in hair bundles.**

Mechanoelectrical transduction by a vertebrate hair cell is initiated by force applied to its receptive organelle, the hair bundle. A bundle comprises dozens to hundreds of stereocilia, cylindrical processes derived from microvilli, which project from the cellular apex in staggered ranks. Deflection of a hair bundle in the excitatory direction opens mechanically sensitive ion channels situated near the stereociliary tips (reviewed in ref. 1). Although K^+ carries most of the transduction current, the channels are also highly permeable to other cations (2, 3). The entry of Ca^{2+} in particular is important because the Ca^{2+} concentration within stereocilia determines the rate and extent of adaptation, the process whereby a hair bundle's position of sensitivity is continuously adjusted. Adaptation is thought to be mediated by a molecular motor that sets the tension in elastic gating springs that open and close the transduction channels (reviewed in ref. 1). Like the transduction channels (4, 5), the machinery responsible for adaptation occurs atop the hair bundle (6).

To promote adaptation, Ca^{2+} must interact with proteins in the hair bundle. A common intracellular receptor for Ca^{2+} is calmodulin, a 17-kDa protein which binds Ca^{2+} and modulates the activity of other proteins (reviewed in ref. 7). The many targets with which calmodulin interacts include protein kinases and phosphatases, phosphodiesterases, adenylyl cyclases, Ca^{2+} pumps, ion channels, myosins, and cytoskeletal proteins. Immunohistochemical and biochemical experiments have demonstrated the presence of calmodulin in hair bundles, where it is concentrated near the stereociliary tips (8).

With the advent of techniques for the purification of hair bundles (8–10) and for the sensitive detection of their protein constituents (11), hair bundles have become amenable to biochemical experimentation. The crucial role of Ca^{2+} in mechanoelectrical transduction and calmodulin's intriguing localization spurred us to determine the role of calmodulin and its receptors in hair bundles. As an initial step toward that end, we have measured the amount of calmodulin present in bundles and developed a calmodulin–alkaline phosphatase conjugate (CaM-AP) with which to identify calmodulin's receptors (12).

MATERIALS AND METHODS

Materials. Bovine brain calcineurin, 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), imidazole, and 2-mercaptoethylamine were purchased from Sigma. ^{125}I-labeled bovine brain calmodulin (≈90 GBq/mol) was obtained from DuPont/NEN. Other materials were obtained from the suppliers indicated in refs. 10 and 11.

Hair-Bundle Isolation and Detergent Extraction. Bullfrog (*Rana catesbeiana*) saccular hair bundles were isolated as described (10) and quantified on the assumption that each complete preparation contained 3000 bundles (10, 13). The subcellular distribution of calmodulin and calmodulin-binding proteins was analyzed as follows. Isolated bundles were permeabilized by freezing and thawing (10) in 7.5 μl of a solution containing 100 mM KCl, 2.5 mM $MgCl_2$, 20 μM $CaCl_2$, 2.5 mM dithiothreitol, 1 μM pepstatin A, 1 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, and 25 mM Hepes at pH 8.0, with or without 1 mM EGTA. To minimize protein loss, we included as a carrier protein either *Escherichia coli* maltose-binding protein (ref. 14; for calmodulin-immunoblotting experiments) or bovine hemoglobin (for protein-detection experiments) at 0.1 mg/ml. After centrifugation at 29,000 × *g* for 15 min, the supernatant was removed and retained for analysis. The insoluble material was extracted for 1 hr with the same solutions containing 1% (vol/vol) Triton X-100 and sedimented as above. Both the supernatant and the pellet were saved for analysis.

Immunoblotting of Hair-Bundle Calmodulin. After separation by SDS/PAGE in 15% acrylamide minigels, calmodulin or hair-bundle proteins were transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore) by 30 min of exposure at 4°C to a field of ≈2200 V/m; the transfer solution contained 2 mM $CaCl_2$ and 10 mM Caps at pH 11.0. Known quantities of bovine brain calmodulin (Calbiochem) were included in the gels as standards. To decrease the loss of calmodulin from the membranes, transferred proteins were fixed with 40 mM glutaraldehyde (15) in a solution containing 68 mM NaCl and 75 mM sodium phosphate at pH 7.4.

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Abbreviations: CaM-AP, calmodulin–alkaline phosphatase conjugate; SATA, *N*-succinimidyl *S*-acetylthioacetate; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

Calmodulin was detected with mouse monoclonal antibodies in blocking solution (11). Quantitative immunoblotting was initially conducted with a commercially available IgG1 (1 μg/ml) directed against a fragment of bovine brain calmodulin (Upstate Biotechnology, Lake Placid, NY); this antibody is reported by the manufacturer to react identically with calmodulins from a variety of vertebrates. The results were confirmed with an IgG raised against bovine testis calmodulin. Detection of bound antibodies and densitometry were performed as described (10, 11).

Synthesis of CaM-AP. The conjugation of calmodulin to alkaline phosphatase was accomplished by first derivatizing the two proteins with complementary reactive groups, then allowing the two proteins to couple. All reactions were conducted at room temperature and all solutions were filtered with 0.2-μm filters.

One milligram of calf intestinal alkaline phosphatase (EC 3.1.3.1; Boehringer Mannheim) was modified with the heterobifunctional protein crosslinking reagent succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; Pierce) at 3 mM in 0.5 ml of a conjugation solution consisting of 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ in 25 mM Hepes at pH 7.4. The SMCC was initially dissolved in dimethylformamide, which was present in the reaction mixture at a concentration of 1.5%. The modification of alkaline phosphatase was allowed to proceed for 1 hr, after which unreacted SMCC was diluted by addition of 2 ml of ice-cold conjugation solution. The product was then concentrated by centrifugation in an ultrafiltration unit (Centricon-30; Amicon), diluted with 2 ml of conjugation solution containing 0.1 mM $CaCl_2$ and 1 mM tris(carboxyethyl)phosphine (a non-sulfhydryl reducing agent; Molecular Probes), and concentrated to a final volume of 100 μl.

Because vertebrate calmodulins contain no cysteine residues (7), we next introduced protected sulfhydryl groups onto some of calmodulin's primary amino groups. One milligram of bovine brain calmodulin was incubated for 30 min in 0.5 ml of a solution containing 0.3 mM *N*-succinimidyl *S*-acetylthioacetate (SATA; Pierce) and 1 mM tris(carboxyethyl)phosphine buffered at pH 7.5 with 25 mM Hepes. The SATA was first dissolved in dimethylformamide, whose concentration in the reaction mixture was 1.5%. To remove unreacted SATA, the reaction mixture was diluted with 2 ml of the ice-cold buffer solution without SATA, concentrated with Centricon-10 units, diluted, and concentrated to a final volume of 100 μl. To deprotect the sulfhydryl moiety introduced onto calmodulin, hydroxylamine was added to a final concentration of 50 mM. This reaction was allowed to proceed for 1 hr. The derivatized calmodulin was then diluted in conjugation solution and concentrated to a volume of 100 μl.

The SMCC-modified alkaline phosphatase and SATA-modified calmodulin were then incubated together for 2 hr, during which time calmodulin's added sulfhydryl groups exerted a nucleophilic attack on alkaline phosphatase's maleimide moieties. To terminate the conjugation reaction, unreacted maleimide groups were quenched with 1 mM 2-mercaptoethylamine. The reaction mixture was subjected to gel-filtration chromatography (tandem Superose 6 HR 10/30 columns; Pharmacia LKB) in a solution containing 100 mM Na_2SO_4, 1 mM $MgSO_4$, 0.1 mM $ZnSO_4$, and 0.1 mM $CaSO_4$ in 25 mM Hepes at pH 7.5. Solvent was delivered with a liquid chromatograph (1090M; Hewlett–Packard) at a flow rate of 0.5 ml/min. The absorbance of the eluant was monitored at 280 nm. CaM-AP emerged as a single broad peak, the first half of which (from the void volume to 150 kDa) was retained for use as a probe of calmodulin-binding proteins. After the protein in this fraction was concentrated to a final volume of 300 μl with Centricon-30 concentration units, glycerol was added to a final concentration of 50% (vol/vol). The final protein concentration was usually ≈0.6 mg/ml. When stored at −20°C, the conjugate was effective for at least 6 months.

Detection of Calmodulin-Binding Proteins. Purified calmodulin-binding proteins or hair-bundle proteins were subjected to SDS/PAGE in minigels and then electrophoretically transferred to charged nylon membranes (10). After transfer, membranes were incubated for 1 hr in 150 mM NaCl buffered at pH 7.5 with 50 mM imidazole. Remaining protein-binding sites on the membranes were saturated for 2–4 hr with a blocking solution containing 6% (wt/vol) casein, 1% (wt/vol) polyvinylpyrrolidone 40, 3 mM NaN_3, 0.1 mM $CaCl_2$, 150 mM NaCl, and 50 mM imidazole at pH 7.5. After blocking, membranes were incubated for 1 hr with blocking solution containing either a 1:1000 dilution of CaM-AP conjugate (a final concentration of 0.6 μg/ml) or 1 μM calmodulin biotinylated to the extent of 1.9 mol/mol with *N*-hydroxysulfosuccinimidobiotin (11).

CaM-AP on membranes was detected after washing with four 5-min changes of 0.3% (vol/vol) Tween 20/0.1 mM $CaCl_2$/150 mM NaCl/50 mM imidazole, pH 7.5, and then with four 5-min changes of 0.1 mM $CaCl_2$/1 mM $MgCl_2$/25 mM Tris, pH 9.5. The membranes were next incubated for 5 min in the latter solution containing the chemiluminescence substrate, 400 μM 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (Tropix, Bedford, MA), and treated as described (10, 11). Biotinylated calmodulin on membranes was detected with streptavidin–alkaline phosphatase conjugate essentially as described (11), except that the solutions used were those detailed for CaM-AP detection.

Detection of ^{125}I-calmodulin-binding proteins was accomplished by using a modified blot-overlay assay (16, 17). Membranes were incubated for 2–4 hr in the imidazole-buffered blocking solution, then probed with ^{125}I-calmodulin (3.7 kBq/ml) for 3 hr. Unbound calmodulin was removed with four 5-min changes of 0.3% Tween 20/0.1 mM $CaCl_2$/150 mM NaCl/50 mM imidazole, pH 7.5. Excess moisture was removed from the membranes by blotting between filter papers. Each membrane was ensheathed in plastic wrap, then exposed for ≈12 hr to a Eu^{2+}:BaFBr phosphor screen (Molecular Dynamics). The exposed screen was scanned (400B PhosphorImager; Molecular Dynamics) and a gray-scale image was produced.

RESULTS

Quantitative Immunoblotting of Hair-Bundle Calmodulin. Although hair bundles are known to contain calmodulin (8), the amount of this Ca^{2+}-binding protein present has not been ascertained. To determine accurately the quantity of calmodulin in the hair bundle, we employed two monoclonal anti-calmodulin antibodies and measured the amount of calmodulin by quantitative immunoblotting. By applying a chemiluminescence detection method, we could detect as little as 1 pg, or 60 amol, of bovine brain calmodulin. Using a mouse monoclonal antibody against a peptide from bovine brain calmodulin, we estimated the total calmodulin content of the hair bundles isolated from each bullfrog's sacculus as 209 ± 20 pg (mean ± SD, n = 5; Fig. 1*A*). A second monoclonal antibody, directed against bovine testis calmodulin, produced an estimate of 174 ± 46 pg (n = 8) for the total calmodulin content (data not shown).

To ascertain the subcellular distribution of calmodulin in hair bundles, we sequentially extracted purified bundles with a buffer solution and with Triton X-100. When the bundles were extracted with a solution containing 1 mM EGTA, over 90% of the calmodulin was soluble (Fig. 1*B*). By contrast, when the bundles were extracted with a solution containing 20 μM Ca^{2+}, at least 50% of the calmodulin remained bound to detergent-soluble intracellular receptors. A small fraction

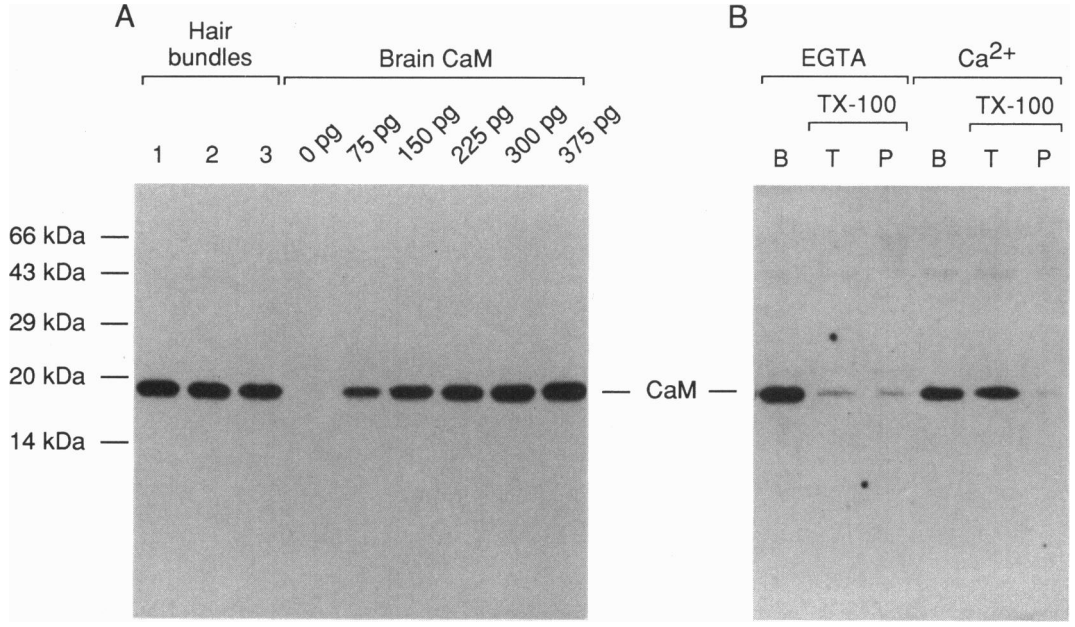

FIG. 1. Hair-bundle calmodulin. (*A*) Quantitative immunoblotting. Calmodulin transferred to and fixed on a membrane was detected with a chemiluminescence assay. Lane 1, ≈5100 hair bundles; lane 2, ≈4800 hair bundles; lane 3, ≈3300 hair bundles; lanes 4–9, the indicated amounts of bovine brain calmodulin (CaM). Hair-bundle calmodulin was quantified by densitometric scanning of autoradiographs and interpolation from a linear standard curve. (*B*) Subcellular distribution. Two preparations, each of ≈4000 isolated hair bundles, were extracted with solutions containing 20 μM Ca^{2+} or 1 mM EGTA. The bundles were extracted sequentially with buffer solution alone and then with the same solution containing 1% Triton X-100 (TX-100) (10). Calmodulin immunoblotting was employed to analyze the supernatant from the extraction with buffer solution (lanes B), that from the Triton X-100 extraction (lanes T), and the final pellet (lanes P).

of the bundles' calmodulin was resistant to extraction with detergent in the presence or absence of Ca^{2+}.

Synthesis of CaM-AP. Calmodulin's presence in hair bundles, and particularly its accumulation near the site of transduction and adaptation, led us to seek receptors for calmodulin in stereocilia. Because the hair bundles from each sacculus contain a total of no more than 40 ng of protein (10), this endeavor required an unusually sensitive assay. Techniques using biotinylated calmodulin (18) and ^{125}I-labeled calmodulin (16, 19) proved insufficiently sensitive, so we developed a conjugate, CaM-AP, that allowed us to take advantage of the sensitivity of chemiluminescence assays.

The conjugation procedure involved the addition of chemically reactive groups to alkaline phosphatase and to calmodulin, followed by reaction of the two activated species to form an intermolecular crosslink. SDS/PAGE disclosed that the resulting conjugate was a heterogeneous mixture; 90% of the products had molecular masses of at least 77 kDa, the size expected for a single calmodulin molecule linked to one alkaline phosphatase molecule. Control experiments established that the conjugation procedure did not diminish alkaline phosphatase's enzymatic activity (data not shown).

CaM-AP Recognizes Calmodulin-Binding Proteins. When the conjugate was used with chemiluminescence detection, it recognized known calmodulin-binding proteins that had been transferred to charged-nylon membranes. For example, the conjugate permitted detection on protein blots of as little as 100 pg, or 2 fmol, of calcineurin (Fig. 2*A*), a Ca^{2+}/calmodulin-activated phosphatase (17, 20). Neither biotinylated calmodulin nor ^{125}I-calmodulin was able to detect <10 ng of calcineurin (Fig. 2 *B* and *C*). As expected in view of the Ca^{2+} sensitivity of the calmodulin–calcineurin interaction (17, 20), the binding of CaM-AP to calcineurin was greatly diminished by 1 mM EGTA (Fig. 2*D*). Binding of CaM-AP to calcineurin was also reduced in the presence of 1 μM bovine brain calmodulin (Fig. 2*E*). No signal was observed when blotted calcineurin was probed with a mixture of unconjugated calmodulin and alkaline phosphatase (data not shown).

Numerous CaM-AP-binding proteins were observed in detergent extracts of bullfrog skeletal muscle, kidney, heart, and brain, as well as in the residual macula, the cellular constituents left in the saccular macula after bundle isolation (Fig. 3*A*). The conjugate also recognized several purified proteins known to bind calmodulin, including chicken p190 myosin, human erythrocyte spectrin, and bovine brain calmodulin-activated phosphodiesterase (data not shown). CaM-AP failed to recognize at least one calmodulin-binding protein in the blot-overlay assay: although the conjugate sensitively detected native bovine adrenal myosin I directly applied to a nylon membrane, CaM-AP failed to recognize this protein after SDS/PAGE and electrophoretic transfer. Because we confirmed that adrenal myosin I was efficiently transferred, we presume that the molecule's calmodulin-binding domains failed to renature on the membrane.

Calmodulin-Binding Proteins in Isolated Hair Bundles. To determine the receptors for calmodulin in stereocilia, we employed CaM-AP to examine hair-bundle proteins that had been electrophoretically transferred to membranes. We observed an ensemble of calmodulin-binding proteins distinct from those in residual maculae. The prominent hair-bundle proteins were those of 25, 35, 145, 175, 240, and 350 kDa (Fig. 3*B*). Additional components of 33, 36, 57, and 107 kDa, as well as several of >350 kDa, appeared in our preparations at lower abundance. Because 1 mM EGTA abolished binding of the probe to all the proteins (Fig. 3*B*), they evidently bound calmodulin in a Ca^{2+}-dependent manner. The interaction of CaM-AP with the proteins was also eliminated by the inclusion of 1 μM calmodulin (Fig. 3*B*).

To determine the subcellular distribution of the calmodulin-binding proteins, isolated hair bundles were extracted with a buffer solution and with the nonionic detergent Triton X-100. Although none of the calmodulin receptors was soluble in the buffer solution, all of the prominent calmodulin-binding proteins save the 240-kDa species were relatively detergent-soluble both in the presence (Fig. 3*C*) and in the absence (data not shown) of Ca^{2+}.

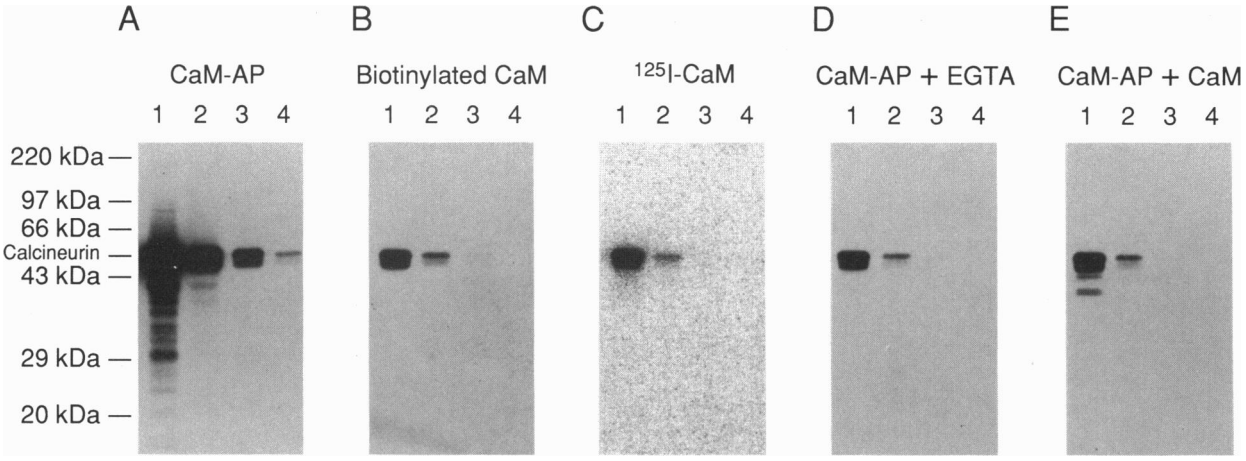

FIG. 2. Comparison of the sensitivities of various methods for detection of calmodulin-binding proteins. Bovine brain calcineurin was subjected to SDS/PAGE in 10% acrylamide gels, transferred to membranes, and detected by use of the following reagents: CaM-AP at 0.6 μg/ml with 100 μM Ca^{2+} (*A*), 10 μM biotinylated calmodulin (CaM) with 100 μM Ca^{2+} (*B*), and ^{125}I-calmodulin at 3.7 kBq/ml with 100 μM Ca^{2+} (*C*). To characterize the binding of CaM-AP to calcineurin, identical blots were incubated with CaM-AP at 0.6 μg/ml in the presence of 100 μM Ca^{2+} and either 1 mM EGTA (*D*) or 1 μM unlabeled calmodulin (*E*). The following amounts of calcineurin were loaded on each gel: lane 1, 100 ng; lane 2, 10 ng; lane 3, 1 ng; lane 4, 100 pg.

DISCUSSION

Use of CaM-AP for Detection of Calmodulin-Binding Proteins. To identify calmodulin-binding proteins in the very limited amount of starting material available from hair cells, we have developed a technique that takes advantage of the sensitivity afforded by chemiluminescence detection. By applying the CaM-AP assay to detergent extracts of several tissues, we observed ensembles of calmodulin-binding proteins that resemble those reported by others (18). Although the hair cells and supporting cells of the bullfrog's sacculus appear to share many calmodulin-binding proteins with other tissues (Fig. 3*A*), CaM-AP recognizes a unique set of proteins in hair bundles (Fig. 3*B*). We identified in hair bundles six prominent and several minor calmodulin receptors, most of which are membrane-associated. Armed with the ability to recognize proteins with which calmodulin interacts, we can investigate the roles of calmodulin's targets in adaptation and in other aspects of the transduction process.

The CaM-AP assay has several virtues that render it of general utility in the recognition of calmodulin-binding proteins. The conjugate is easily synthesized in 1 day with

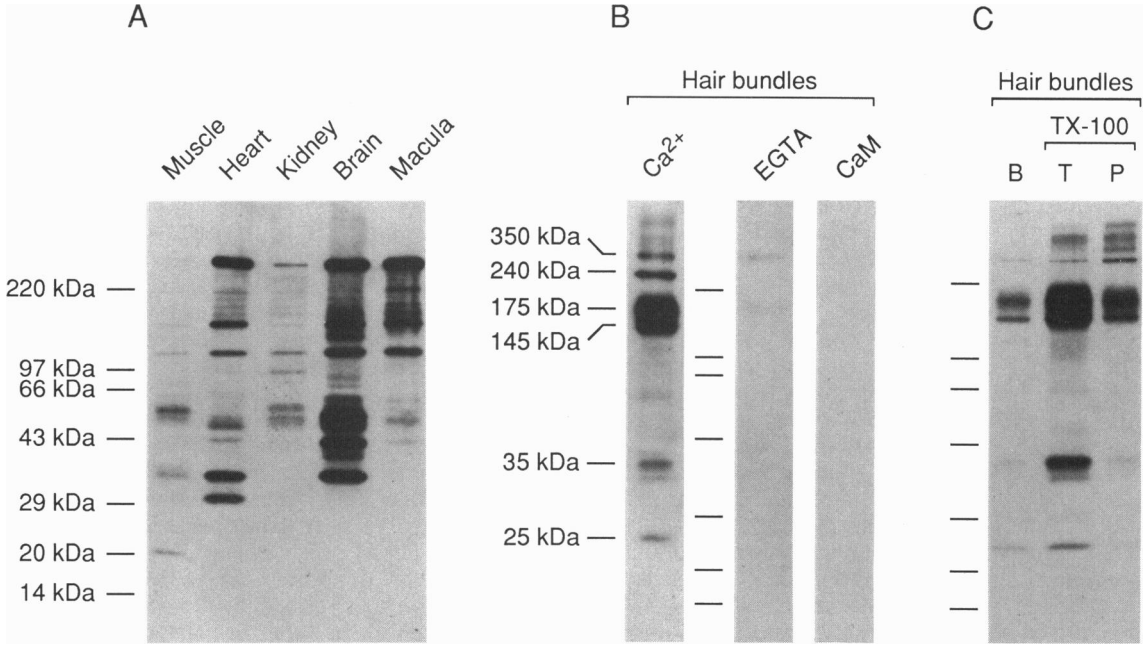

FIG. 3. Detection of calmodulin-binding proteins with CaM-AP. (*A*) CaM-AP detection of calmodulin-binding proteins in detergent extracts of skeletal muscle, heart, kidney, and brain, as well as in residual macula. Each sample contained 1 μg of protein. (*B*) Calmodulin-binding proteins of the hair bundle. Three samples, each of ≈30,000 hair bundles, were subjected to SDS/PAGE, transferred to a nylon blotting membrane, and detected with CaM-AP in the presence of 100 μM Ca^{2+}, 1 mM EGTA, or 100 μM Ca^{2+} and 1 μM unlabeled calmodulin (CaM). (*C*) Subcellular distribution of calmodulin-binding proteins in hair bundles. A preparation of ≈18,000 isolated bundles was extracted with solutions containing 20 μM Ca^{2+}. The bundles were extracted sequentially with buffer solution alone and then with the same solution containing 1% Triton X-100 (TX-100) (10). CaM-AP in the presence of 100 μM Ca^{2+} was used to analyze the supernatant from the extraction with buffer solution (lane B), that from the Triton X-100 extraction (lane T), and the final pellet (lane P). The unlabeled molecular-mass indicators in *B* and *C* corresponded to those in *A*.

readily available reagents. Because no radionuclides are used in the assay, hazards for the investigator are greatly reduced. Moreover, unlike a radioactive probe's activity, that of CaM-AP does not appreciably diminish over time. Whereas conventional gel-overlay methods require several days, the CaM-AP assay takes less than a day. The binding of CaM-AP to receptors exhibits the desirable characteristics of appropriate Ca^{2+} dependence and sensitivity to excess calmodulin. Most importantly, the CaM-AP procedure is ≈100-fold more sensitive than conventional methods for detecting calmodulin-binding proteins.

Calmodulin in Hair Bundles. Our immunoblotting results demonstrate that the ≈3000 hair bundles of a bullfrog's sacculus contain a total of 11 fmol of calmodulin, or ≈4 amol per hair bundle. A single stereocilium thus embraces ≈40,000 calmodulin molecules. The concentration of calmodulin in cells and organelles varies widely: photoreceptor outer segments, for example, contain only 4 μM calmodulin (21), while the concentration exceeds 1 mM in intestinal microvilli (22). Because the cytoplasmic volume of each hair bundle in the bullfrog sacculus is ≈60 fl (13), the average calmodulin concentration in each stereocilium of a bundle is ≈70 μM. Immunohistochemical analysis indicates that the concentration is still greater at the stereociliary tips (8), the site of mechanoelectrical transduction (4, 5) and adaptation (6).

When the Ca^{2+} concentration in stereocilia is low, calmodulin should be largely dissociated (Figs. 1*B* and 3*B*) from its membrane-associated receptors (Fig. 3*C*). Although calmodulin would then be free to diffuse, its accumulation at the hair bundle's top suggests that much of the calmodulin is bound to receptors there. Studies with Ca^{2+}-sensitive dyes indicate that the average concentration of free Ca^{2+} in unstimulated hair bundles is <100 nM (unpublished observations). The Ca^{2+} concentration may be locally greater, however, adjacent to the transduction channels: even when a bundle is at rest, these channels open sporadically and admit Ca^{2+} near the stereociliary tips. Perhaps calmodulin accumulates at a bundle's top because only there is the Ca^{2+} concentration great enough to load calmodulin's binding sites and thus to promote its association with receptors.

We thank Ms. L. Henry for technical assistance and Mr. R. A. Jacobs for help with preparation of the figures. Dr. B. Barylko provided a sample of adrenal myosin I, Dr. J. Beavo a sample of phosphodiesterase, Dr. M. Mooseker a sample of p190 myosin, and Dr. J. T. Stull an anti-calmodulin antibody. For comments on the manuscript, we thank the members of our laboratory group and Drs. J. P. Albanesi, S. T. Brady, J. A. Mercer, and W. J. Snell. This research was supported by National Institutes of Health Grant DC00241.

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