Specific Localization of Scallop Gill Epithelial Calmodulin in Cilia

E. W. STOMMEL, R. E. STEPHENS, H. R. MASURE, and J. F. HEAD
Marine Biological Laboratory, Woods Hole, Massachusetts 02543; and the Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118

ABSTRACT Calmodulin has been isolated and characterized from the gill of the bay scallop *Aequipecten irradians*. Quantitative electrophoretic analysis of epithelial cell fractions shows most of the calmodulin to be localized in the cilia, specifically in the detergent-solubilized membrane-matrix fraction. Calmodulin represents 2.2 ± 0.3% of the membrane-matrix protein or 0.41 ± 0.05% of the total ciliary protein. Its concentration is at least 10^{-4} M if distributed uniformly within the matrix. Extraction in the presence of calcium suggests that the calmodulin is not bound to the axoneme proper. The ciliary protein is identified as a calmodulin on the basis of its calcium-dependent binding to a fluphenazine-Sepharose affinity column and its comigration with bovine brain calmodulin on alkaline-urea and SDS polyacrylamide gels in both the presence and absence of calcium. Scallop ciliary calmodulin activates bovine brain phosphodiesterase to the same extent as bovine brain and chicken gizzard calmodulins. Containing trimethyllysine and lacking cysteine and tryptophan, the amino acid composition of gill calmodulin is typical of known calmodulins, except that it is relatively high in serine and low in methionine. Its composition is less acidic than other calmodulins, in agreement with an observed isoelectric point ~0.2 units higher than that of bovine brain. Comparative tryptic peptide mapping of scallop gill ciliary and bovine brain calmodulins indicates coincidence of over 75% of the major peptides, but at least two major peptides in each show no near-equivalency. Preliminary results using ATP-reactivated gill cell models show no effect of calcium at micromolar levels on ciliary beat or directionality of the lateral cilia, the cilia which constitute the vast majority of those isolated. However, ciliary arrest will occur at calcium levels >150 μM. Because calmodulin usually functions in the micromolar range, its role in this system is unclear. Scallop gill ciliary calmodulin may be involved in the direct regulation of dynein-tubule sliding, or it may serve some coupled calcium transport function. At the concentration in which it is found, it must also at least act as a calcium buffer.

There is substantial evidence for the existence of calmodulin in cilia and flagella. However, its specific localization and function in these organelles is uncertain. Satir and co-workers (20; see also reference 17) find *Tetrahymena* calmodulin to be concentrated considerably more in cilia than in the cell body, using immunofluorescent techniques, whereas Walter and Schultz (27) find a greater concentration of *Paramecium* calmodulin in cell bodies than in cilia, using SDS-polyacrylamide gel analysis. Gitelman and Witman (10) report that calmodulin from *Chlamydomonas* occurs in both the cell body and in isolated flagella, with the latter appearing to contain a much greater concentration of calmodulin. About half of the flagellar calmodulin remains tightly bound to the axoneme after detergent removal of the membrane-matrix fraction, implying specific binding. Reed and Satir (19) find a calmodulinlike protein in the ciliated epithelium of a fresh water mussel gill but do not report its relative distribution.

Calcium control of ciliary beating is documented in many organisms. Using cell models of *Paramecium*, Naitoh and Eckert (18) find that cilia on the anterior portion beat normally at a calcium concentration of 10^{-7} M but reverse their direction at 10^{-5} M. Machemer and Ogura (16) report voltage-sensitive and mechanosensitive calcium channels in the ciliary membrane and the somatic membrane, respectively, of *Paramecium*. Satir (21) can induce ciliary arrest in the lateral cilia of fresh water mussel gills by perfusion with millimolar-levels of Ca^{++}

THE JOURNAL OF CELL BIOLOGY · VOLUME 92 MARCH 1982 622-628

in the presence of the ionophore A23187. Tsuchiya (25), using reactivated cell models of the salt water mussel *Mytilus*, can arrest the lateral cilia by raising the calcium levels from 10^{-7} M to 10^{-6} M. Walter and Satir (26) obtain similar results from models of exfoliated lateral cells of a fresh water mussel, but they note that considerably higher levels of calcium are needed to arrest frontal and laterofrontal cells. The ciliary arrest caused by increased calcium levels can be reversed by applying trifluoperazine (19); this drug binds to calmodulin, inactivating it.

With regard to the possible function of calmodulin in cilia, Blum and co-workers (4) report various degrees of enhancement of *Tetrahymena* dynein ATPase activity, depending upon the extraction medium, by adding bovine brain calmodulin. They are able to reverse the calmodulin-induced enhancement by chlorpromazine treatment. Thus dynein-dependent microtubule interaction may be regulated by calmodulin.

In this report, we isolate, characterize, and quantitate a unique calmodulin from the ciliated epithelium of the scallop, demonstrate its specific localization in the ciliary membrane-matrix fraction, and suggest several alternatives for its function.

MATERIALS AND METHODS

Gill Epithelium Fractionation

Typically, 100–150 g of gills from the bay scallop *Aequipecten irradians* were fractionated into cilia and free, deciliated epithelial cells (mainly frontal and lateral)[1] by the hypertonic salt method of Stephens (22, 23). In most cases, phenylmethylsulfonylfluoride (PMSF), at a concentration of 0.1 g/liter, was included in all steps except the actual deciliation. In other cases, isotonic, buffered NaCl was substituted for 10 mM Tris-HCl, pH 8.0, in the differential centrifugation steps to avoid osmotic shock to the isolated cilia.

Preparation of Calmodulin

A fluphenazine-Sepharose 4B affinity column (1.5 × 7.5 cm) was prepared by the method of Charbonneau and Cormier (6). The column was washed and equilibrated with 10 mM imidazole, 200 mM NaCl, 1 mM $CaCl_2$, and 15 mM 2-mercaptoethanol, pH 7.1. Cilia were extracted with 5–10 vol of 0.25% Nonidet P-40 (NP-40), 3 mM $MgCl_2$, 30 mM Tris-HCl (pH 8.0), and PMSF (0.1 g/liter) at 0°C for 30 min and then centrifuged at 25,000 *g* for 15 min. The supernatant, made 1 mM in $CaCl_2$, was applied to the column and nonadhering proteins were removed by washing with at least 10 column vol of equilibration buffer. Calmodulin was eluted with 10 mM imidazole, 5 mM EGTA, 200 mM NaCl, and 15 mM 2-mercaptoethanol, pH 7.1. Column fractions were analyzed by alkaline-urea polyacrylamide gels (see below) and the calmodulin-containing fractions were pooled, dialyzed exhaustively against 0.1 M ammonium bicarbonate, 15 mM 2-mercaptoethanol, and lyophilized. Bovine brain and chicken gizzard calmodulins were prepared essentially as described by Head et al. (13).

PAGE

A continuous polyacrylamide (8T/2.5C) system containing 8 M urea and buffered with 3.0 g Tris base and 6.0 g glycine/liter was used to monitor column fractions (12). For quantitation of calmodulin in cell fractions, the discontinuous SDS system of Laemmli (15) was modified to include a 5–15% polyacrylamide gradient as the resolving gel. Identical samples containing either 2.5 mM $CaCl_2$ or 2.5 mM EGTA were run in alternate lanes of a slab gel to detect mobility shifts in the calmodulin due to calcium binding (10). For accurate quantitation, gels were stained with Fast Green (0.1–0.25%) in 10% acetic acid and either 40% methanol (2–4 h) or 25% isopropanol (overnight). For detection of minor impurities, the Coomassie Blue method of Fairbanks and coworkers (8) was used. Fast Green stained gels were scanned at 650 nm on an Ortec 5310 densitometer, using known amounts of bovine brain calmodulin as a calibration standard. Quantitation was by either peak-weight or planimetry. Isoelectric focusing was by the method of Berkowitz and coworkers (3), here using a pH 4–6 ampholyte (Bio-Rad Laboratories, Richmond, Calif.); direct staining was with Crocein Violet/Coomassie Blue (Bio-Rad, unpublished method).

[1] In the *Pectinidae*, the true laterofrontal ciliated cells are diminutive ("micro-laterofrontal") and are few in number. The frontal cells also bear short cilia and occupy a comparatively small area of the gill filament. The lateral ciliated cells, bearing long cilia, are by far the dominant form in gills of these species (2). Because of the marked differences between the ciliature of *Aequipecten* and the widely studied mussel gill, the isolated cells of *Aequipecten* gill were previously misidentified as being mainly frontal (22).

Phosphodiesterase Assay

Phosphodiesterase activity was determined by the method of Boudreau and Drummond (5). Samples of bovine brain and chicken gizzard calmodulins of known concentration were used as standards in activation studies.

Amino Acid Analysis

The scallop ciliary calmodulin was hydrolyzed in 6 N HCl for 24, 48, and 96 h at 110°C and analyzed on a Durrum D-500 single-column amino acid analyzer (AAA Laboratory, Mercer Island, Wash.). Serine and threonine were extrapolated to zero hydrolysis time. Trimethyllysine was detected as a discrete peak, leading lysine.

Tryptic Peptide Mapping

Ciliary and bovine brain calmodulins were digested for 24 h at 25°C with DCC-treated trypsin (Calbiochem-Behring Corp., La Jolla, Calif.) at a 1:100 enzyme:protein ratio. A second addition of enzyme was made and the digestion was continued for an additional 6 h at 37°C. The resultant peptides (1–1.5 nmol; 17–25 μg) were spotted on Silica Gel GHL thin-layer plates (Analtech, Inc., Newark, Del.) and subjected to ascending chromatography with chloroform-methanol-ammonium hydroxide (2:2:1, vol/vol), followed by perpendicular electrophoresis at pH 3.5 with pyridine-acetic acid-water (1:10:489). The resolved peptides were sprayed with fluorescamine and visualized with 366 nm illumination. Details of this procedure appear elsewhere (24).

Gill Epithelium Reactivation

Excised strips of gill filaments, or free epithelial cells released from the gill by EDTA treatment (22), were reactivated as cell models by the method of Tsuchiya (25). Calcium control of ciliary arrest was investigated, using the Tsuchiya "arrest-reactivating solution" containing calcium chloride in the range of 0.05 to 1.0 mM. In living cell experiments, the ionophore A23187 (Calbiochem-Behring Corp.) at 10^{-5} M in sea water was added to excised strip gills in the presence of either 10 mM $CaCl_2$ or 10 mM EGTA in artifical sea water. The effect of fluphenazine, trifluoperazine, and chlorpromazine was tested at concentrations of 50–200 μM in normal sea water.

RESULTS

Purity of Fractions

A fraction of pure cilia, devoid of contaminating epithelial cells or nuclei, is obtained after two sets of 1,000 *g*/10,000 *g* differential spins (Fig. 1*A*). The epithelial cell pellet, after two 1,000 *g* isotonic washes, consists of totally deciliated cell bodies, free of contaminating cilia (Fig. 1*B*). Because the vast majority of the ciliated cells of the scallop gill is comprised of frontal and lateral cells, and because both cell types are released upon treatment with high salt, the preparation should consist mainly of those two cell types. In fact, the majority appear to be lateral cells, as is evident from their flat, columnar appearance (Fig. 1*B*, arrows).

Detection and Quantitation of Calmodulin

Equal weights (or volumes, because pellet densities were the same) of purified cilia and deciliated epithelial cells were extracted twice for 15 min with 10 vol of NP-40 extraction buffer, each extraction being followed by a 25,000 *g* centrifugation. The extracted pellets were resuspended in an equivalent volume of NP-40 extraction buffer. Duplicate samples, containing either Ca^{++} or EGTA, were applied to alternate wells

of a Laemmli SDS-polyacrylamide gradient gel, flanked by bovine brain calmodulin as a mobility-shift standard. Typical results of such a stoichiometric assay are shown in Fig. 2.

A calmodulinlike protein is found in the first detergent extract of cilia (CS_1) but none is evident in the second extract (CS_2), the "9+2" axoneme pellet (*Axo*), the first or second extract of the epithelial cells (ES_1 and ES_2), or the extracted epithelial cell pellet (*EP*). The detectability of calmodulin is enhanced by the marked mobility shift between and across adjacent lanes, containing either calcium or EGTA. This "cross-over" phenomenon between lanes aids in visual detection because the (minor) calmodulin bands no longer run in parallel with the other proteins in the same mol wt region.

Simple graphical integration of densitometric scans from ciliary membrane-matrix supernatant and extracted axoneme samples (not run in the above alternating fashion) allows an accurate estimation of the amount of calmodulin present (Fig. 3). The ciliary calmodulinlike protein represents 2.2 ± 0.3% (SD, n = 8) of the membrane-matrix fraction (first detergent extract) or 0.41 ± 0.05% (SD, n = 4) of the whole organelle (membrane-matrix plus axoneme). At our limits of detection, which are about tenfold lower than the amounts of calmodulinlike protein determined in the first detergent extract, we could detect no calmodulin in the deciliated epithelial cell extracts. The same results were obtained whether cilia and epithelial cells were prepared by the previously published hypotonic methods (22, 23) or whether all steps were isotonic, as was the case in Fig. 2. Because both PMSF and EGTA were included in the preparative and extraction steps of the experiment illustrated, digestion of epithelial cell calmodulin by either a trypsin-like or calcium-activated protease is unlikely. Neither substitution of Triton X-100 for NP-40 nor inclusion of millimolar levels of calcium in the extraction medium affects the concentration of calmodulinlike protein extracted from the cilia in the membrane-matrix fraction (Fig. 4).

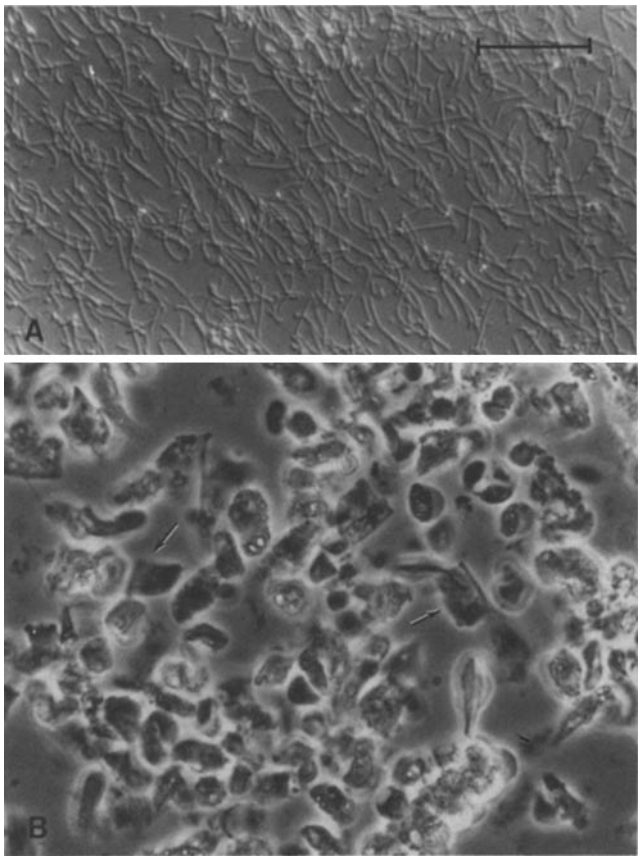

FIGURE 1 Purified cilia (*A*) and deciliated epithelial cells (*B*) from the gill of *Aequipecten irradians*. The epithelial cells are mainly lateral (*B*, arrows). Differential-interference (*A*) and phase contrast (*B*). Bar, 25 μm.

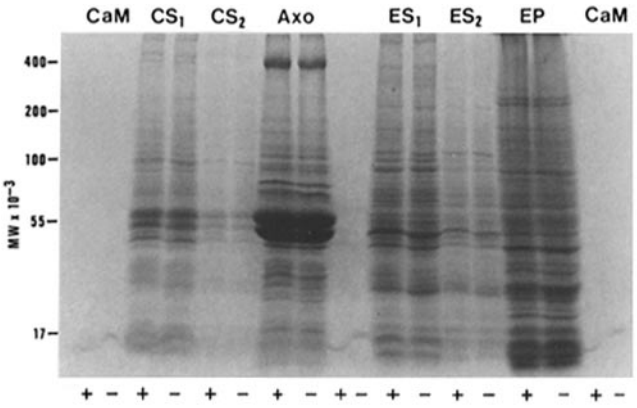

FIGURE 2 Stoichiometric SDS-polyacrylamide gel analysis of ciliary and epithelial cell extracts. Extractions were performed with equal amounts of cilia and cells, using 10 vol of 0.25% NP-40 extraction buffer containing 1.0 mM EGTA. CS_1 and CS_2: first and second ciliary supernatants; ES_1 and ES_2: first and second epithelial cell supernatants. Axo and EP are the resultant axoneme and epithelial cell pellets, taken up in a volume of buffer equal to the original extraction volume. CaM, bovine brain calmodulin. Equal sample volumes, containing either 2.5 mM $CaCl_2$ (+) or 2.5 EGTA (−), were applied. Fast Green staining.

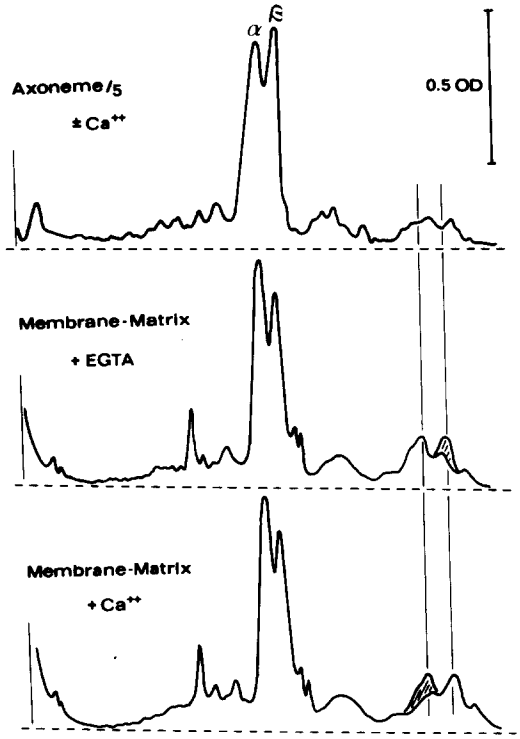

FIGURE 3 Densitometer scans of ciliary axoneme and membrane-matrix fractions run in the presence or absence of calcium. The axoneme sample was loaded at ⅕ the stoichiometric ratio for comparison. The shaded regions in the membrane-matrix scans reflect the amount of calmodulin present because these are differences between the scans due to the calmodulin shift. Fast Green staining, scanned at 650 nm.

Fluphenazine Affinity Purification

When the detergent-solubilized ciliary membrane-matrix fraction is passed over a fluphenazine-Sepharose 4B affinity column, most of the protein remains unbound. Elution with an EGTA-containing buffer releases a single polypeptide that comigrates with bovine brain calmodulin on alkaline-urea gels (Fig. 5, *left panel*). Unlike *Chlamydomonas* flagellar calmodulin (10), moderate salt concentrations are required for release of the protein from the column by EGTA.

Isoelectric focusing of the column-purified ciliary protein reveals one major species with a pI of 4.45 and a minor species (9–10% by densitometry) with a pI equal to that of bovine brain calmodulin, 4.25 under our conditions. Because the pH gradient at this extreme pH is nonlinear, these values must be taken as approximate, but relative to bovine brain calmodulin, the ciliary counterpart is 0.2 units less acidic (Fig. 5, *right panel*).

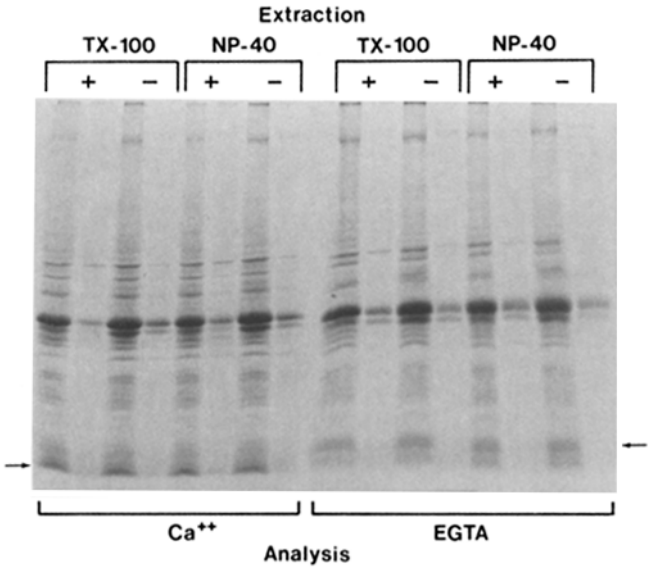

FIGURE 4 Ciliary membrane-matrix extraction with either Triton X-100 or Nonidet P-40. Extractions were performed with either 1.0 mM $CaCl_2$ (+) or 1.0 mM EGTA (−) in the extraction buffer and then analyzed with either 2.5 mM $CaCl_2$ (*left set*) or 2.5 mM EGTA (*right set*) added to the gel samples. Lane pairs contain first and second supernatants. Arrows mark calmodulin positions. Coomassie Blue staining.

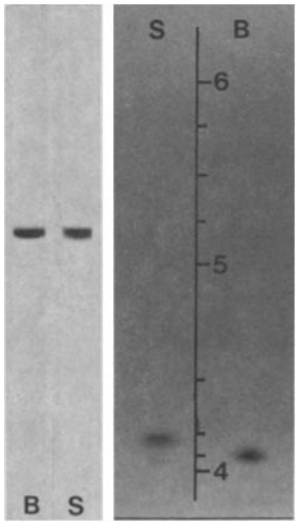

FIGURE 5 Purity of ciliary calmodulin from a fluphenazine affinity column. Alkaline-urea gels (*left panel*) and isoelectric focusing (*right panel*) compare bovine brain calmodulin (*B*) with scallop ciliary calmodulin (*S*). Coomassie Blue and Crocein Violet/Coomassie Blue staining, respectively.

Phosphodiesterase Activation

Scallop gill ciliary calmodulin activates bovine brain phosphodiesterase to exactly the same extent and over the same range of protein concentration as does chicken gizzard calmodulin (Fig. 6) or bovine brain calmodulin (data not shown). The scallop calmodulin shows no activation in the presence of 1 mM EGTA or 200 μM trifluoperazine; its activation of phosphodiesterase is approximately halved by the inclusion of 300 mM NaCl in the assay mixture.

Amino Acid Composition

Table I summarizes the amino acid composition of scallop gill ciliary calmodulin in comparison with the range in composition reported for various vertebrate and invertebrate calmodulins (14). With the exception of major differences in two amino acids, the scallop protein has the amino acid composi-

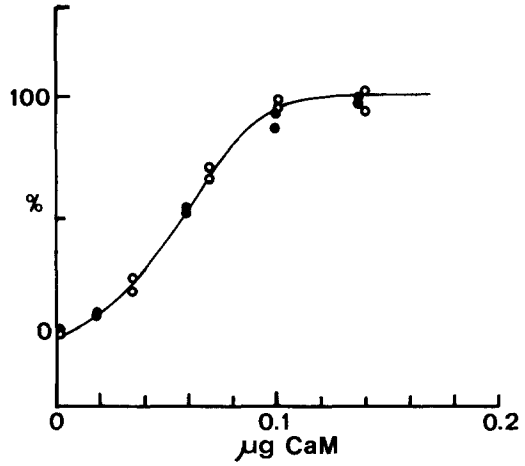

FIGURE 6 Activation of bovine brain phosphodiesterase by scallop ciliary and chicken gizzard calmodulins. Open circles: scallop; filled circles: gizzard.

TABLE I

Amino Acid Composition of Scallop Gill Calmodulin (S-CaM) Compared with Known Calmodulins (CaM's; from Jamieson et al., 1980. Annu. N.Y. Acad. Sci.).

	S-CaM	CaM's
Ala	11.0 ± 0.4	10–11
Arg	5.6 ± 0.4	5–6
Asx	21.6 ± 1.1	23–27
Cys	0	0
Glx	22.3 ± 1.2	25–27
Gly	13.6 ± 0.4	11–12
His	1.2 ± 0.1	1
Ilu	6.3 ± 0.2	6–9
Leu	8.9 ± 0.2	8–9
Lys	7.2 ± 0.1	7–8
Met	3.7 ± 0.4	8–11
Phe	8.3 ± 0.3	8–9
Pro	2.8 ± 0.4	2–3
Ser	11.5 ± 0.6	4–5
Thr	11.0 ± 0.6	10–12
Try	0	0
Tyr	1.5 ± 0.1	1–2
Val	7.7 ± 0.3	6–7
Tml	1.0 ± 0.1	1

Data expressed as residues/mol (16,700) of protein. Average of three determinations, ±SD. *Ser* and *Thr* were extrapolated to zero hydrolysis time; ± 5%. *Met*: Met plus methionine sulfoxide. *Tml*: trimethyllysine.

tion typical of a calmodulin, including the presence of one trimethyllysine and the absence of cysteine and tryptophan. The serine is comparatively high, by a factor of two, but this deviation is not unique because a similar value has been reported for sea urchin egg calmodulin (13). The methionine content is low, again by a factor of two, even though our analyses include methionine sulfoxide in the total. The sea urchin egg counterpart is also lower in methionine than the typical calmodulin and both it and the ciliary calmodulin are marginally high in glycine and valine. The ciliary protein is lower than the bovine brain protein of by six residues of Asx plus Glx; if any of these are the acids rather than the amides, one would expect the ciliary protein to have a somewhat higher isoelectric point than that from bovine brain, as observed in our isoelectric focusing studies (Fig. 5).

Comparative Peptide Mapping

When tryptic peptide maps of ciliary calmodulin are compared with those of bovine brain, at least 13 major peptides are coincident (Fig. 7). One intense peptide in brain calmodulin (*) appears to have a counterpart in ciliary calmodulin which splits into two peptides of approximately half the intensity. Two major peptides (*B*) are unique to brain calmodulin while four major peptides (*S*) are unique to the scallop protein. Reproducible differences occur in the relative intensity of the minor peptides. From the amino acid composition, one would predict a maximum of 15 major tryptic peptides for scallop ciliary calmodulin. Because more than this are found, some must represent partial digestion products, in spite of the double digestion used for both proteins.

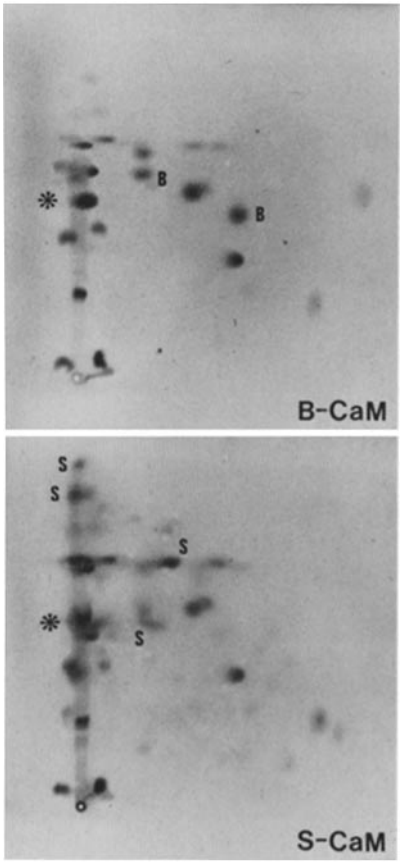

FIGURE 7 Comparative tryptic peptide maps of bovine brain and scallop ciliary calmodulins. Major peptides unique to brain (*B*) and scallop (*S*) are marked; one equivalent peptide (*) splits in the scallop sample.

Reactivation Studies

Scallop gill cilia may be readily reactivated *in situ* by the methods of Tsuchiya (25). Brief (15–30 s) extraction at 22°C with low concentrations (0.010–0.012%) of Triton X-100 will stop cilia of the lateral cells. Higher concentrations of detergent or longer extraction times are required to stop the frontal cilia, to the detriment of the rest of the tissue. The lateral ciliated cells remain relatively intact and may be reactivated to nearly 100% upon perfusion with ATP; even when tissue disintegration occurs, these cells are readily distinguished. Because the lateral cells and cilia constitute the bulk of our starting material for the above biochemical studies, and because these cells are stable and easily reactivated, we concentrated primarily on them.

The lateral cilia generally beat continuously in excised tissue but they are apparently under neuronal control and will occasionally arrest, in a down stroke, spontaneously. They may be stopped at will by perfusion with dopamine (10 μM) in sea water (cf. 1). We found no evidence of calcium-induced arrest in the lateral cells using either cell models with 10–50 μM calcium (as in reference 25; with *Mytilus*) or perfusing living tissue with the ionophore A23187 (as in reference 21; with fresh water mussel).

To determine to what extent calmodulin might be retained in the cilia of such permeabilized cells, we extracted isolated cilia for 30 min at 4°C with 10 vol of the Tsuchiya (25) extracting solution and then reextracted these cilia twice more with the same vol of our normal NP-40 extraction buffer. The results of such an experiment, run stoichiometrically, are shown in Fig. 8. Although there is some solubilization of membrane components, no detectable calmodulin appears to "leak out". We can be reasonably confident that our cell models (which

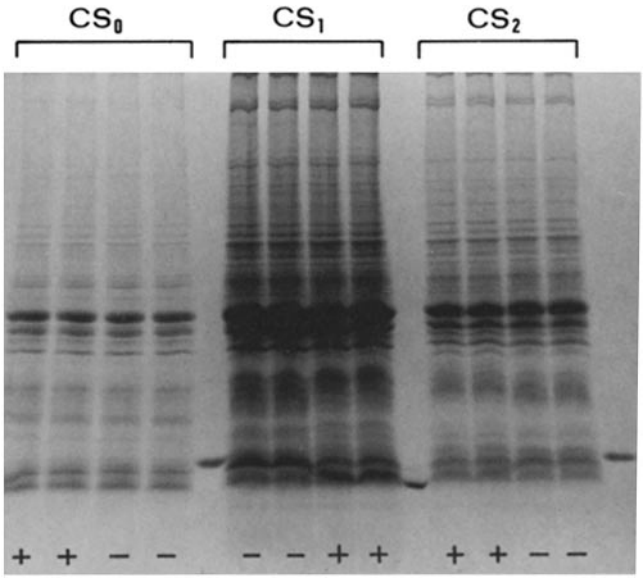

FIGURE 8 Extraction of isolated cilia with an extraction-reactivation buffer to test for calmodulin release. Cilia were extracted with 10 vol of Tsuchiya (1977) extraction buffer and the supernatant (CS_0) retained. The pellet was then reextracted twice with a 0.25% NP-40 extraction buffer (CS_1 and CS_2). Run as paired, duplicate samples in the presence (+) or absence (−) of calcium, flanked by brain calmodulin as a standard. Coomassie Blue staining.

show no micromolar-level calcium sensitivity under standard reactivating and arrest conditions) still retain the bulk of their ciliary calmodulin.

Full arrest of the lateral cilia, mimicking the natural arrest, can be achieved by perfusion of reactivating solutions containing calcium in excess of 150 μM. The arrest is fully reversible by subsequent perfusion of reactivating solution having lower or no added calcium. Addition of trifluoperazine (200 μM) to the reactivating solution containing threshold levels of calcium will also restore motility. Perfusion of living tissue with trifluoperazine (and related compounds) at 200 μM has no overt effect on ciliary motility except that spontaneous arrest appears to be more frequent and metachrony is poor. At substantially higher concentrations (0.5–1.0 mM), rapid deciliation and mucus discharge occur.

Restoration of calcium arrested movement with trifluoperazine may not be due to inactivation of calmodulin with the drug. At the minimal calcium concentration required to arrest movement, 150 μM, the trifluoperazine forms a cloudy suspension. Because we must exceed this calcium concentration with trifluoperazine to see any effect, we may be complexing calcium, lowering the effective concentration below the threshold needed for ciliary arrest.

DISCUSSION

The calcium-binding protein isolated from scallop gill cilia is a bona fide calmodulin based upon currently accepted criteria. It has the same mol wt (M_r, 16,700), undergoes the same electrophoretic shift upon binding calcium, and migrates on alkaline-urea gels with the same mass/charge parameters as bovine brain calmodulin. The protein binds to a fluphenazine-Sepharose affinity column in a calcium-dependent manner and it will activate mammalian brain phosphodiesterase to the same extent as either vertebrate brain or muscle calmodulin. This activation is totally abolished with an excess of either EGTA or trifluoperazine. Scallop ciliary calmodulin can be distinguished from bovine brain calmodulin on the basis of significant differences in the otherwise characteristic amino acid composition and tryptic peptide map, and also by a pI 0.2 units more basic than brain calmodulin.

Scallop ciliary calmodulin comprises ~0.4% of the protein of the organelle, but no comparable concentration can be detected in the cell body. Assuming 50 cilia, 20 μm in length, on a cell 5 × 10 × 10 μm, one calculates a volume ratio of 1:16. If the same amount of calmodulin occurs in the cell body as in the cilia, we would not detect it because we can detect only about 1/10 the amount of calmodulin that we quantitate from cilia, using Coomassie Blue staining and scale expansion. If the same concentration of calmodulin occurs in the cell body as in the cilia, it would be detectable at the same level as in the cilia because the gel analyses were performed stoichiometrically. Thus we conclude that calmodulin may occur in the epithelial cell body but at no greater than 1/10 the concentration that we find in cilia.

Scallop ciliary calmodulin appears to occur in concentrations equal to or greater than those found in other systems. Gitelman and Witman (10) find that *Chlamydomonas* flagellar calmodulin accounts for 0.6% of the membrane-matrix fraction and 0.3% of the axoneme; depending upon total protein concentration, the calmodulin concentration in the cell bodies (7 μg/ml) is at least one order of magnitude lower. Walter and Schultz (27) report that *Paramecium* calmodulin accounts for only 50 μg/g ciliary protein, with the cell body having a 50% higher concentration, a value probably comparable to *Chlamydomonas*.

By densitometry, we find a 1:100 weight ratio of calmodulin to axonemal tubulin, allowing us to calculate not only mole ratios to various axonemal proteins but also the minimal concentration of calmodulin in the cilium. Taking a 240 Å thick cross-section (i.e. one dynein arm repeat unit), we calculate that there are 45 calmodulins: one for every 15.5 tubulin dimers and 2.5 for every dynein arm.[2] If uniformly distributed within the volume of the cross-section, the concentration of calmodulin would be 10^{-4} M.[3] Because the "9+2" structure must occupy some of this volume, to the exclusion of calmodulin, the concentration of calmodulin must be even greater.

There are three obvious functions that may be suggested for ciliary calmodulin. The first is direct regulation of sliding. The above stoichiometric calculations would allow at least two calmodulins for every dynein arm complex and even more for every spoke head. However, the fact that ciliary calmodulin may be so readily extracted in the presence of calcium makes this possibility less attractive, unless it regulates through some very weak interaction. Another possibility is that calmodulin may be a part of a membrane Ca^{++}-ATPase system, similar to that found in erythrocytes (e.g. reference 11). Here, the function would be to bind calcium ions and then interact with a membrane Ca^{++}-ATPase which, in turn, would pump the calcium ions out of the cilium. The presence of a Ca^{++}-ATPase in the membrane fraction of cilia (7, 9) supports such a contention. Finally, the high concentration of calmodulin would suggest that the protein must act as a "calcium sink", regardless of what other functions might be ascribed to it. This effect may explain why such high initial calcium levels are required to induce ciliary arrest in salt water molluscan gill cell models. One might speculate that the differential response of various molluscan gill epithelial cell types to changes in intracellular calcium levels is governed by the relative amount of calmodulin within their cilia.

This work was supported by National Institutes of Health grants GM 20,644; GM 29,503; and AM 18,207; and by National Science Foundation grant PCM 79-04600.

Received for publication 29 September 1981.

REFERENCES

1. Aiello, E. 1974. Control of ciliary activity in metazoa. *In*: Cilia and Flagella. M. A. Sleigh, editor. Academic Press, Inc., New York. 353–376.
2. Atkins, D. 1938. On the ciliary mechanisms and interelationships of Lamellibranchs. Part VII. Latero-frontal cilia of the gill filaments and their phylogenetic value. *Q. J. Microsc. Sci.* 80:345–436.
3. Berkowitz, S. A., J. Katagiri, H. K. Binder, and R. C. Williams, Jr. 1977. Separation and characterization of microtubule proteins from calf brain. *Biochemistry.* 16:5610–5617.
4. Blum, J. J., A. Hayes, G. A. Jamieson, and T. C. Vanaman. 1980. Calmodulin confers calcium sensitivity on ciliary dynein ATPase. *J. Cell Biol.* 87:386–397.

[2] In a 240 Å cross-section, there are nine doublets of 23 protofilaments each and two central tubules with 13 each, all three dimers (3 × 80 Å) in length, for a total of 699 tubulin dimers and 18 dynein arms. Because the weight ratio of CaM: tubulin is 1:100, the mole ratio is 1:15.5 (assuming mol wt of 17,000 and 110,000 respectively), there are 45 calmodulins (one for every 15.5 dimers) and 2.5 calmodulins per morphological dynein arm.

[3] In a 1 μm length of cilia, there are 29,125 tubulin dimers (using the parameters in footnote 2), or 927,548/μm^3, assuming a diameter of 0.2 μm. Using Avogadro's Number, this amounts to the equivalent of 1.54 × 10^{-3} M tubulin, if it were uniformly distributed throughout the volume. Because there is a 15.5:1 mole ratio between tubulin and calmodulin, the calmodulin concentration would be 1 × 10^{-4} M.

5. Boudreau, R. J., and G. I. Drummond. 1975. A modified assay of 3':5'-cyclic-AMP phosphodiesterase. *Anal. Biochem.* 63:388–399.
6. Charbonneau, H., and M. J. Cormier. 1979. Purification of plant calmodulin by fluphenazine-Sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.* 90:1039–1047.
7. Doughty, M. J., and S. Dryl. 1981. Control of ciliary activity in paramecium: and analysis of chemosensory transduction in a eukaryotic unicellular organism. *Prog. Neurobiol. (N. Y.)* 16:1–115.
8. Fairbanks, G. T., and T. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocytes. *Biochemistry.* 10:2606–2617.
9. Gibbons, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* 76:317–352.
10. Gitelman, S. E., and G. B. Witman. 1980. Purification of calmodulin from *Chlamydomonas*: calmodulin occurs in cell bodies and flagella. *J. Cell Biol.* 98:764–770.
11. Graf, E., and J. T. Penniston. 1981. Equimolar interaction between calmodulin and the Ca^{2+} ATPase from human erythrocyte membranes. *Arch. Biochem. Biophys.* 210:257–262.
12. Head, J. F., and S. V. Perry. 1974. The interaction of the calcium-binding protein (trophonin C) with bivalent cations and the inhibitory protein (troponin I). *Biochem. J.* 137:145–154.
13. Head, J. F., S. Mader, and B. Kaminer. 1979. Calcium-binding modulator protein from the unfertilized egg of the sea urchin *Arbacia punctulata. J. Cell Biol.* 80:211–218.
14. Jamieson, G. A., D. D. Bronson, F. H. Schachat, and T. C. Vanaman. 1980. Structure and function relationships among calmodulins and troponin C-like proteins from divergent eukaryotic organisms. *Ann. N. Y. Acad. Sci.* 356:1–13.
15. Laemmli, Y. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature (Lond.).* 227:680–685.
16. Machemer, H., and A. Ogura. 1979. Ionic conductances of membranes in ciliated and deciliated *Paramecium. J. Physiol.* 296:49–60.
17. Maihle, N. J., J. R. Dedman, A. R. Means, J. G. Chafouleas, and B. R. Satir. 1981. Presence and indirect immunofluorescent localization of calmodulin in *Paramecium tetraurelia. J. Cell Biol.* 89:695–699.
18. Naitoh, Y., and R. Eckert. 1974. The control of ciliary activity in protozoa. *In*: Cilia and Flagella. M. A. Sleigh, editor. Academic Press, Inc., New York. 305–352.
19. Reed, W., and P. Satir. 1980. Calmodulin in mussel gill epithelial cells: role in ciliary arrest. *Annu. N. Y. Acad. Sci.* 356:423–426.
20. Satir, B., R. S. Garofalo, D. M. Gilligan, and N. J. Maihle. 1980. Possible functions of calmodulin in protozoa. *Annu. N. Y. Acad. Sci.* 356:83–91.
21. Satir, P. 1975. Ionophore-mediated calcium entry induces mussel gill ciliary arrest. *Science* (Wash. D. C.). 190:586–588.
22. Stephens, R. E. 1975. The basal apparatus. Mass isolation from the molluscan ciliated gill epithelium and a preliminary characterization of striated rootlets. *J. Cell. Biol.* 64:408–420.
23. Stephens, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry.* 16:2047–2058.
24. Stephens, R. E. 1978. Fluorescent thin-layer peptide mapping for protein identification and comparison in the subnanomole range. *Anal. Biochem.* 84:116–126.
25. Tsuchiya, T. 1977. Effects of calcium ions on triton-extracted lamellibranch gill cilia: ciliary arrest response in a model system. *Comp. Biochem. Physiol.* 56A:353–361.
26. Walter, M. F., and P. Satir. 1978. Calcium control of ciliary arrest in mussel gill cells. *J. Cell Biol.* 79:110–120.
27. Walter, M. F., and J. E. Schultz. 1981. Calcium receptor protein calmodulin isolated from cilia and cells of *Paramecium tetraurelia. Eur. J. Cell Biol.* 24:97–100.