Specific Localization of Scallop Gill Epithelial Calmodulin in Cilia

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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++.
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 tri-
fluoperazine (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) 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 x 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, containing either Ca$^{++}$ or EGTA, were applied to alternate wells of a slab gel to detect mobility gradient as the resolving gel. Identical samples containing either 2.5 mM CaCl$_2$ or 10 mM Tris-HCl, pH 7.1. Column fraction was 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).

**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 the Coomassie Blue method of Fairbanks and coworkers (8) was used. Fast Green stained gels were scanned at 650 nm on an Ortec 5330 densitometer, using known amounts of bovine brain calmodulin as a calibration standard. Quantitation was by either peak-weigh or planimetry. Isotopic focusing was used 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/ Cilium Calmodulin
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 calmodulin-like protein is found in the first detergent extract of cilia (CS₁) but none is evident in the second extract (CS₂), the "9+2" axoneme pellet (Axo), the first or second extract of the epithelial cells (ES₁ and ES₂), 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 calmodulin-like 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 calmodulin-like 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 calmodulin-like protein extracted from the cilia in the membrane-matrix fraction (Fig. 4).
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).

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-

| 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    |
| Ile   | 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; ±3%. 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.

**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

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**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.

**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 (CS0) retained. The pellet was then reextracted twice with a 0.25% NP-40 extraction buffer (CS1 and CS2). 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 pM 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 mu-
tober more frequent and metachrony is poor. At substantially

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 complicating calmodulin 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.

For the above reasons we conclude that calmodulin may occur in the epithelial cell body but at no greater than 1/60 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 *Paramaecium* calmodulin accounts for only 50 μg/g ciliary protein, with the cell body having a 50% higher concentration, a value probably comparable to *Chlamydo-

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.

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5 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 Ca:M; 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.

5 In a 1 μm length of cilia, there are 29,125 tubulin dimers (using the parameters in footnote 2), or 927,548/μm², assuming a diameter of 0.2 μm. Using Avogadro's Number, this amounts to the equivalent of 1.54 × 10⁻⁸ 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 × 10⁻⁷ M.

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