The cilia of *Paramecium tetraurelia* contain both Ca^{2+} -dependent and Ca^{2+} -inhibitable calmodulin-binding proteins

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To identify protein targets for calmodulin (CaM) in the cilia of *Paramecium tetraurelia*, we employed a ^{125}I -CaM blot assay after resolution of ciliary proteins on SDS/polyacrylamide gels. Two distinct types of CaM-binding proteins were detected. One group bound ^{125}I -CaM at free Ca^{2+} concentrations above 0.5–1 μM and included a major binding activity of 63 kDa (C63) and activities of 126 kDa (C126), 96 kDa (C96), and 36 kDa (C36). CaM bound these proteins with high (nanomolar) affinity and specificity relative to related Ca^{2+} receptors. The second type of protein bound ^{125}I -CaM only when the free Ca^{2+} concentration was below 1–2 μM and included polypeptides of 95 kDa (E95) and 105 kDa (E105). E105 may also contain Ca^{2+} -dependent binding sites for CaM. Both E95 and E105 exhibited strong specificity for *Paramecium* CaM over bovine CaM. Ciliary subfractionation experiments suggested that C63, C126, C96, E95, and E105 are bound to the axoneme, whereas C36 is a soluble and/or membrane-associated protein. Additional Ca^{2+} -dependent CaM-binding proteins of 63, 70, and 120 kDa were found associated with ciliary membrane vesicles. In support of these results, filtration binding assays also indicated high-affinity binding sites for CaM on isolated intact axonemes and suggested the presence of both Ca^{2+} -dependent and Ca^{2+} -inhibitable targets. Like E95 and E105, the Ca^{2+} -inhibitable CaM-binding sites showed strong preference for *Paramecium* CaM over vertebrate CaM and troponin C. Together, these results suggest that CaM has multiple targets in the cilium and hence may regulate ciliary motility in a complex and pleiotropic fashion.

INTRODUCTION

Ca^{2+} plays a central role as a messenger that regulates several cellular functions in many organisms. For well-studied cases, Ca^{2+} acts at multiple levels; it regulates some cellular processes both directly and indirectly through feedback control and by interacting with other second-messenger systems (reviewed in [1]). Despite recent progress, the molecular pathways that underlie these events remain incompletely understood.

In the unicellular protozoan *Paramecium*, Ca^{2+} is a key regulator of cell motility (reviewed in [2]). Under resting conditions, *Paramecium* cells swim forward by the coordinated beating of thousands of cilia. Depolarization of the cell membrane by various stimuli opens voltage-gated Ca^{2+} channels in the ciliary membrane, causing Ca^{2+} influx into the cilia. Ca^{2+} then triggers reversal of the ciliary-beat direction, resulting in backward swimming [3]. Ciliary-reversal episodes are transient; cells recover as the membrane potential and/or Ca^{2+} levels return to resting values.

As in other systems, the regulation of *Paramecium* motility by Ca^{2+} is complex. In addition to mediating ciliary reversal, Ca^{2+} also regulates the duration and extent of its own entry by inactivating Ca^{2+} channels [4], and by activating Ca^{2+} -dependent K^{+} channels [5] and Ca^{2+} -dependent Na^{+} channels [6], which influence membrane potential (see [7] for a review). In addition, recent observations suggest that both cyclic AMP and cyclic

GMP regulate ciliary-beat frequency and beat orientation [8–10] and that Ca^{2+} interacts with these pathways [8,10–13]. Ca^{2+} may also stimulate ciliary-beat frequency [14,15]. The regulation of ciliary motility in *Paramecium* therefore shares many characteristics with other flagellar-based motility systems (reviewed in [16,17]) and other Ca^{2+} -regulated processes. Furthermore, genetic, physiological and biochemical approaches can be combined in *Paramecium* to provide a powerful system for gaining insight into the molecular mechanisms of Ca^{2+} -dependent regulation.

The molecular targets and mechanisms of Ca^{2+} action on motility are largely unknown. However, several observations suggest that the Ca^{2+} -binding protein calmodulin (CaM) may be involved in one or more of the Ca^{2+} -dependent functions in *Paramecium*. CaM is present in cilia [18,19], and the effects of CaM antagonists on the swimming behaviour of permeabilized cells are consistent with some role for CaM in the regulation of ciliary reversal and/or the axoneme's response to cyclic AMP [20,21]. Moreover, recent evidence strongly implicates CaM in the regulation of the Ca^{2+} -dependent K^{+} current; the phenotype of a mutant, *pntA*, lacking this current can be rescued by microinjected wild-type CaM [22], and *pntA* CaM contains a single amino acid change [23]. In other organisms, CaM is a component of cilia and flagella [24–27], and purified CaM has been shown to affect the beat properties of extracted sperm flagella [28].

Abbreviations used: CaM, calmodulin; BSA, bovine serum albumin; MLCK, myosin light-chain kinase; PDE, phosphodiesterase; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis; CMV, ciliary membrane vesicles; IDC, incompletely demembrated cilia; IC_{50} , concentration causing 50% inhibition; C-proteins, Ca^{2+} -dependent calmodulin-binding proteins.

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As a step toward understanding how CaM regulates ciliary function at the molecular level, we have initiated studies of CaM-binding proteins in *Paramecium* cilia. Our results suggest that CaM may have multiple targets in cilia, including axonemal proteins, soluble proteins and membrane-associated proteins. In addition to several Ca^{2+} -dependent CaM-binding proteins, we report the finding of an unusual type of protein that binds CaM only when free Ca^{2+} concentrations are below the micromolar range. These polypeptides are associated with the axoneme and exhibit strong specificity for *Paramecium* CaM over closely related proteins. Thus CaM may regulate several activities in cilia, inducing distinct physiological responses as Ca^{2+} concentrations change during and after membrane excitation. Together with available assays for ciliary motility [10,29] and genetic analyses [7], these studies provide a framework for gaining insight into the physiological function of CaM and its targets in *Paramecium*.

EXPERIMENTAL

Materials

Purified troponin C from rabbit skeletal muscle was generously given by Dr. M. Greaser (University of Wisconsin). Rabbit skeletal-muscle myosin light-chain kinase (MLCK) was generously provided by Dr. James Stull (University of Texas Southwestern Medical Centre). Parvalbumin, melittin and bovine serum albumin (BSA) were from Sigma. ^{125}I -labelled Bolton–Hunter reagent was from ICN Radiochemicals. All chemicals used were of reagent grade. CNBr-activated Sepharose was from Pharmacia, and phenyl-Sepharose was from Sigma. Melittin-Sepharose resin was prepared essentially as in [30]. Molecular-mass standards were myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase *b* (92.5 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and α -lactalbumin (14 kDa), as specified by the supplier (Bio-Rad).

CaM preparations and iodinations

Two protocols were used to purify *Paramecium* CaM. In method I, CaM was purified from strain-51s whole cells by monoclonal-antibody affinity chromatography as described [31]. CaM purified by this method was > 99% homogeneous, activated MLCK and brain PDE [31] and has been sequenced [23,32]. In method II, CaM was purified from whole cells by a modification of the procedure used for purification of *Paramecium* Ca^{2+} -dependent protein kinase II [33]. The high-speed supernatant of cell homogenates was applied to a phenyl-Sepharose column in the presence of Ca^{2+} , and the EGTA eluate was then applied to an h.p.l.c. anion-exchange column (Mono Q) and developed with a NaCl gradient [33]. CaM was eluted from Mono Q by a 1 M-NaCl wash, dialysed against buffer I (20 mM-Hepes/500 mM-NaCl/0.2 mM- CaCl_2 /0.02% NaN_3 , pH 7.2) and further purified by affinity chromatography on melittin-Sepharose by modification of the procedure in [30]. The CaM was eluted from melittin-Sepharose with buffer II (buffer I with 2 mM-EDTA and no added Ca^{2+}) and then concentrated and dialysed against 20 mM-Tris/0.2 mM- CaCl_2 /0.02% NaN_3 , pH 7.5. This CaM was the same as CaM derived from method I in: (1) amino acid composition, (2) recognition by anti-CaM monoclonal antibodies, (3) mobility in SDS/polyacryl-

amide gels and (4) binding to CaM-binding proteins and axonemes. CaM from method I was used in most experiments, and every experiment was performed at least once with this CaM. CaM concentrations were determined by amino acid analysis (University of California Biotechnology Center, Riverside, CA, U.S.A.), or by quantitative enzyme-linked immuno-adsorbent assay [31]. Bovine testis calmodulin was purified as described in [34]. Bovine CaM and troponin C concentrations were determined by amino acid analysis.

CaM purified by method I was iodinated to 600–1200 Ci/mmol (approx. 0.3–0.6 mol of ^{125}I /mol of CaM) by the Bolton–Hunter reagent [35], essentially as in [36]. ^{125}I -CaM co-migrated with unlabelled *Paramecium* CaM after SDS/PAGE and displayed an identical shift in mobility when Ca^{2+} was included in SDS sample buffer (results not shown). Furthermore, ^{125}I -CaM bound MLCK in a Ca^{2+} -dependent and melittin-inhibitable manner as assayed by ^{125}I -CaM blot (results not shown). No proteins other than CaM were detected in labelled preparations.

Cell and ciliary preparations

Wild-type (51s) cells were grown to early stationary phase in wheat grass medium (Pines International) 'bacterized' with *Enterobacter aerogenes* as described [12,37] and were deciliated by minor modifications of the Ca^{2+} -shock procedure [38]. A buffer containing 20 mM-Hepes, 250 mM-sucrose, 3 mM-NaCl and 1 mM-EDTA (pH adjusted to 7.2 with Tris) was used to immobilize cells. Deciliation was achieved by adding KCl and CaCl_2 to 30 mM and 10 mM respectively. Cilia were collected and washed in cilia buffer (20 mM-Hepes/20 mM-KCl/1 mM-EDTA/250 mM-sucrose/0.02% NaN_3 /0.2 mM-PMSF/leupeptin (5 $\mu\text{g}/\text{ml}$), pH 7.2) by centrifugation for 20 min at 27000 *g*. Cilia were either used immediately or were stored at -20°C in 40% (v/v) glycerol in cilia buffer. The CaM-binding protein pattern did not change substantially upon storage at -20°C , except that easily dissociable proteins were lost upon re-centrifugation of cilia stored in this way. No significant difference in CaM-binding activity was seen in cilia collected from cells that were grown axenically [33] (results not shown). All cilia and ciliary fractions were kept at 4°C unless otherwise specified.

Ciliary subfractionations

Freshly prepared cilia were demembranated on ice for 15 min by gentle repeated vortex-mixing in 1% (v/v) Triton X-100 in HEMK buffer [20 mM-Hepes/1 mM-EDTA/2 mM- MgCl_2 /20 mM-KCl/0.2 mM-PMSF/leupeptin (5 $\mu\text{g}/\text{ml}$), adjusted to pH 7.2 with NaOH] at 1–2 mg of protein/ml. Axonemes were separated from the membrane/matrix fraction by centrifugation at 4°C in a Beckman Microfuge and washed twice in HEMK buffer. Alternatively, cilia were demembranated by hypo-osmotic shock as described in [38], except that the extraction buffer was 1 mM-Hepes/0.1 mM-EDTA (pH 7.2)/0.2 mM-PMSF/leupeptin (5 $\mu\text{g}/\text{ml}$).

Either intact or demembranated cilia were subfractionated by centrifugation on discontinuous 20/45/55/66% (w/w) sucrose gradients as described in [38], except that HEMK buffer was used in all solutions. By this method, ciliary membrane vesicles (CMV) sediment to the 20/45% interface, incompletely demembranated cilia

(IDC) band at the 45/55 % interface, and axonemes band at the 55/66 % interface [38]. All cilia and subciliary fraction preparations were routinely observed by phase-contrast microscopy. For some experiments, axonemes were extracted further by incubating them at 2–4 mg/ml in HEMK buffer with 0.6 M-NaCl or 0.6 M-KI for 30 min with occasional gentle vortex-mixing. Insoluble material was removed by centrifugation at 100 000 *g* in a Beckman Airfuge or by ultracentrifugation for 1 h at 40 000 rev./min in a Beckman SW50.1 rotor. Protein concentrations were determined by the method of Lowry [39] with modifications [40], with BSA as a standard.

Two types of trichocyst preparations were used. (1) Purified trichocysts, isolated by discontinuous-sucrose-density-gradient centrifugation [38], were kindly provided by Dr. J. Peterson (University of Wisconsin-Madison). (2) Partially purified trichocysts were prepared as follows. Before cell deciliation, the fluffy layer on top of cell pellets from the first 4 °C wash was removed (see [38]), diluted to 100 ml with HEK (cilia buffer without sucrose) and spun at 27 000 *g* for 20 min. The loose pellet material was washed once in 100 ml of HEK. This fraction contained mostly trichocysts, as judged by phase-contrast microscopy and SDS/PAGE. CaM immunoblots (see below) failed to detect any endogenous CaM in this fraction (results not shown).

¹²⁵I-CaM blot assay

Protein samples were either boiled for 5 min in SDS sample buffer [50 mM-Tris/5 % (w/v) SDS/5 % (v/v) β -mercaptoethanol/10 % (v/v) glycerol/0.003 % Bromophenol Blue, pH 6.8] or were precipitated with 10 % (w/v) trichloroacetic acid and deoxycholate (0.2 mg/ml), washed once with acetone, and then boiled for 5 min in SDS sample buffer. No difference in results was seen between these methods. Protein samples then were resolved by discontinuous SDS/PAGE using the method of Laemmli [41]. For some experiments, a modified Laemmli buffer system was used. The modified system differed in that stacking gels contained 100 mM-Tris and resolving gels contained 250 mM-Tris. Data generated from the modified system are noted as such in Figure legends, and the original system was used in all other experiments.

Resolving gels were either 7 % (w/v) acrylamide or 6–10 %-(w/v)-acrylamide gradients, with 3 % stacking gels. The ¹²⁵I-CaM blot method was modified from Saitoh & Schwartz [42] and Flanagan & Yost [43]. After separation on gels, proteins were transferred electrophoretically to nitrocellulose membranes (Bio-Rad; 0.45 μ m pore size) by modification of the method of Towbin [44]. Transfers were performed at 6 V/cm for 13 h in 25 mM-Tris/192 mM-glycine/20 % (v/v) methanol/0.1 % SDS, pH 8.6. Under these conditions, most of the dynein heavy chains and I-antigen was transferred to the nitrocellulose membranes. Resulting protein blots were washed five times for 20 min in buffer A (20 mM-Hepes/200 mM-NaCl/0.02 % NaN₃) and then blocked for 1–2 h in buffer A with 3 % (w/v) BSA and 0.05 % (v/v) Tween-20 (Sigma). Blots then were incubated with 0.2–5 nM-¹²⁵I-CaM in either buffer C (buffer A with 1 mM-CaCl₂ and 0.05 % Tween-20) or buffer E (buffer A with 1 mM-EGTA/0.05 % Tween-20) at 25 °C for 1.5–2 h. All incubation solutions contained 0.1 % BSA. Blots were washed for 40 min in six changes of the same buffers used for incubations, then were dried and exposed to

preflashed Kodak XAR film at –70 °C with or without intensifying screens (du Pont) [45]. Binding reached apparent equilibrium by 1 h. The relative amount of ¹²⁵I-CaM bound to individual proteins was estimated in some experiments with a scanning laser densitometer (Biomed Instruments).

For some experiments, the free Ca²⁺ concentration was varied in the micromolar range, either by adding CaCl₂ to buffer E or by adding calcium acetate to buffer HE (20 mM-Hepes/180 mM-KCl/5 mM-HEDTA/0.02 % NaN₃/0.05 % Tween-20, pH adjusted to 7.2 with KOH). Mg²⁺/HEDTA buffers were made by adding magnesium acetate to buffer HE. All buffers were adjusted to pH 7.2. The free Ca²⁺, Mg²⁺ and chelator concentrations were calculated by using a computer program as previously described [12]. Metal–ligand affinity constants were those given by Martell & Smith [46]. For the Ca²⁺/HEDTA buffers, free Ca²⁺ concentrations were also measured with a Ca²⁺-sensitive electrode [47] and were in good agreement with calculated values.

¹²⁵I-CaM binding to intact axonemes

For binding experiments, axonemes were prepared by extraction of freshly prepared cilia in 1 % Triton X-100 in HMK-C buffer (20 mM-Hepes/2 mM-MgCl₂/20 mM-KCl/1 mM-EGTA, pH 7.2, with CaCl₂ added to give 50–100 μ M-free Ca²⁺) for 15 min at 4 °C. Axonemes were washed once in HMK-C, once for 45 min in HMK-E (HMK-C except without CaCl₂) and twice more in HMK-E by centrifugation for 5 min in a Beckman Microfuge. Then axonemes were resuspended to 1 mg/ml in either HMK-C or HMK-E with 1 mg of BSA/ml at 4 °C. All axoneme preparations were monitored for integrity by phase-contrast microscopy. Binding reactions were initiated by the addition of ¹²⁵I-CaM (2–3000 c.p.m./ μ l, 1–250 nM-final concn.) and were allowed to proceed at 25 °C for 60–90 min. At indicated times, 20 μ l aliquots were withdrawn and filtered through 0.22 μ m-pore-size nitrocellulose filters (Millipore) that had been pre-soaked for 1 h in buffer A with 3 % BSA and 0.05 % Tween-20. Filters were immediately washed with 5 ml of the same solution used in the incubation and radioactivity was counted in a Packard γ -radiation counter. For every experiment, blanks were run identically, except that axonemes were excluded, and the resulting radioactivity was subtracted from test values. Blank values were generally less than 15 % of test values. Binding equilibrium was reached by 45 min, and binding was stable for at least 2 h (results not shown). The extent of binding was not substantially different with 4 °C incubations (results not shown). Storage of axonemes at 4 °C for more than 48 h resulted in up to a 50 % decrease in binding activity (results not shown).

CaM determination by immunoblot

To determine amounts of endogenous CaM in ciliary and axoneme preparations, immunoblots were performed using monoclonal anti-CaM antibodies. Protein samples were resolved by SDS/PAGE (12.5 or 15 % resolving gels) and transferred to nitrocellulose (0.1 μ m pore size; Schleicher und Schuell) for 2 h at 4 V/cm in 25 mM-Tris/192 mM-glycine/20 % (v/v) methanol. Blots were fixed in 0.25 % glutaraldehyde for 45 min, blocked in 3 % BSA in buffer A, and incubated for 8–13 h in tissue-culture supernatants of monoclonal antibody 5C5 against CaM [31]. Blots were washed four times for 20 min in

TBS (Tris-buffered saline; 20 mM-Tris/150 mM-NaCl/0.2 mM- CaCl_2 /0.02 % NaN_3 , pH 8.0), incubated in goat anti-mouse IgG linked to alkaline phosphatase for 2 h (1:1000 in TBS), washed in TBS and stained in 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml)/NitroBlue Tetrazolium (0.15 mg/ml)/0.7 M-Tris, pH 9.5 [48]. The amounts of CaM in samples were estimated by comparison of immunoreactivity with known amounts of purified CaM run on the same gel.

RESULTS

After treatment of *Paramecium* cells with a Ca^{2+} -shock procedure, cilia can be removed and separated from the cell bodies [38]. The pattern of ciliary proteins separated by SDS/PAGE was characteristically dominated by three major species: the axonemal proteins tubulin and dynein heavy chains, and the I-antigen, which is associated with the ciliary membrane [38] (Fig. 1a, lane 2; see also Fig. 5a below). To detect potential protein targets for CaM action in *Paramecium* cilia, proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes, and then assayed for binding to ^{125}I -CaM. When the ciliary, cell body and deciliation supernatant fractions were assayed in the presence of Ca^{2+} , several CaM-

binding activities appeared to be enriched in cilia (Fig. 1b). The most prominently labelled polypeptide had an M_r of 63 000 (C63). In addition, polypeptides of 126 (C126), 96 (C96) and 36 (C36) kDa were labelled. A 36 kDa CaM-binding protein was also detected in the deciliation supernatant. Other polypeptides labelled to a lesser extent in cilia included at least five bands between 70 and 110 kDa, a doublet at 45–48 kDa, and a 35 kDa protein. Interestingly, two ciliary polypeptides of 95 and 105 kDa (E95, E105) were consistently labelled in solutions containing no Ca^{2+} and excess EGTA (Fig. 1b, lane 5). By contrast, none of the three major protein species of cilia bound ^{125}I -CaM significantly. This pattern of ciliary CaM-binding proteins was very reproducible in more than 15 separate ciliary preparations, although some variation in the relative amounts of the minor activities was observed. Although numerous additional CaM-binding activities were detected in cell bodies (Fig. 1b), we have concentrated on characterization of the ciliary proteins.

Ca^{2+} -dependence and -sensitivity

Most ciliary CaM-binding proteins, including C63, C126 and C36, bound CaM Ca^{2+} -dependently; binding was not observed when excess EGTA was included in the

Fig. 1. CaM-binding proteins of cilia, cell bodies and deciliation supernatant

Cilia were separated from cell bodies as described in the text, and freshly prepared aliquots were added rapidly to boiling SDS sample buffer. Deciliation supernatant was precipitated in trichloroacetic acid and boiled for 5 min in SDS sample buffer. All samples were resolved by SDS/PAGE on a 6–10 % resolving gel and assayed by ^{125}I -CaM blot. (a) Shows the Coomassie Blue stain of the nitrocellulose blot: lane 1, cell bodies (100 μg); lane 2, cilia (100 μg); lane 3, deciliation supernatant (40 μg). In this gel, I-antigen and dynein heavy chains ran together as noted (I + D). Tubulin (T) also is noted. In (b) an autoradiograph of a ^{125}I -CaM blot of cell bodies (lanes 1 and 4), cilia (lanes 2 and 5) and deciliation supernatant (lanes 3 and 6) is shown. The blots were incubated with ^{125}I -CaM and 1 mM- CaCl_2 (buffer C) (lanes 1–3) or 1 mM-EGTA (buffer E) (lanes 4–6). Protein amounts are the same as in (a). The asterisks mark the following CaM-binding polypeptides, in order of increasing mobility; C126, C96, C63 and C36 (in lane 2); E105 and E95 (in lane 5). Abbreviation: MM, molecular mass; S, standards.

Fig. 2. Ca^{2+} -dependence of ^{125}I -CaM binding to ciliary proteins

Cilia were resolved by SDS/PAGE on a 6–10% gel, using a modified Laemmli gel buffer system, and transferred to nitrocellulose. The blot was washed, blocked, and cut into strips, which were incubated with ^{125}I -CaM (0.4 nM) in buffer E (1 mM-EGTA) and various amounts of CaCl_2 , and washed in the same buffers. An autoradiograph of the blot is shown. The calculated free Ca^{2+} concentrations were: lane 1, 1 nM; lane 2, 10 nM; lane 3, 60 nM; lane 4, 100 nM; lane 5, 500 nM; lane 6, 1.0 μM ; lane 7, 2.5 μM ; lane 8, 10 μM ; lane 9, 100 μM ; lane 10, 1 mM. Similar results were obtained using Ca^{2+} /HEDTA buffers for which the calculated free Ca^{2+} concentrations were confirmed by a Ca^{2+} electrode. Only those proteins from 45 to 160 kDa are shown. With this buffer system, E95 is resolved from other CaM-binding proteins, whereas C96 and C105 are poorly resolved from each other.

incubation (Fig. 1). When the Ca^{2+} -dependence of binding was tested using Ca^{2+} /EGTA buffers, these polypeptides bound ^{125}I -CaM at free Ca^{2+} concentrations between 0.5 and 1 μM or greater (Fig. 2), values within the range expected for physiological responses. C36 also bound ^{125}I -CaM with a Ca^{2+} -sensitivity similar to that of other C-proteins (results not shown). It is noteworthy that the Ca^{2+} -sensitivity of binding varied among some of these proteins (e.g., C126 compared with C75 in Fig. 2). This may reflect differences in their affinity for CaM or it may depend on which conformer of Ca^{2+}_n -CaM ($n = 1-4$) is preferred for interaction.

Among the ciliary proteins that bound ^{125}I -CaM in the absence of Ca^{2+} , E95 and E105 were most prominent (Fig. 1b, lane 5). Additional species were detectable, but usually required extended exposures of the autoradiograms. Interestingly, examination of the Ca^{2+} -dependence of these interactions revealed that E95, and probably E105, bound ^{125}I -CaM more poorly at Ca^{2+} concentrations greater than 2 μM . In several experiments, such as that shown in Fig. 2, E95 was clearly resolved from other CaM-binding proteins and exhibited binding only below 2.5 μM -free Ca^{2+} . The failure of E95 to bind ^{125}I -CaM at higher Ca^{2+} concentrations cannot be explained by competition for the label by the Ca^{2+} -dependent CaM-binding proteins, since the total ^{125}I -CaM activity was not substantially depleted during the incubations (results not shown). Furthermore, when blots of whole cilia were labelled with ^{125}I -CaM in excess chelator (HEDTA), and then washed with solutions containing different Ca^{2+} concentrations, label was lost from both E95 and E105 at free Ca^{2+} concentrations of 1.5 μM or greater (Fig. 3). Ca^{2+} removed approx. 70% of the ^{125}I -CaM in the experiment shown in Fig. 3. In separate experiments, Ca^{2+} released up to 90% of the label. Mg^{2+} was ineffective at removing ^{125}I -CaM from these proteins (Fig. 3). Comparison of the results obtained with the Ca^{2+} /HEDTA and Mg^{2+} /HEDTA buffers revealed that the loss of label did not correlate with the calculated free or complexed chelator concentrations; it was specific for Ca^{2+} and not dependent

on the presence of chelator *per se*. Therefore CaM binding to E95 and E105 is inhibitable by Ca^{2+} , in striking contrast with most known CaM-protein interactions [49].

In Fig. 2, E105 appears to bind ^{125}I -CaM Ca^{2+} -independently. One possibility is that there are two polypeptides of this size, one that is Ca^{2+} -dependent (C105), and one that is Ca^{2+} -inhibitable (E105). This is supported by three observations: (1) Ca^{2+} stimulated the release of ^{125}I -CaM from E105, as described above; (2) bovine CaM displaced ^{125}I -CaM from C105 at nanomolar concentrations, but was a weak competitor of binding to E105 (see below); (3) melittin, a potent inhibitor of Ca^{2+} -dependent CaM-protein interactions, blocked binding to C105 but not to E105. Alternatively, it could be that E105 and C105 represent one protein species with two classes of CaM-binding sites: one that is Ca^{2+} -inhibitable

Fig. 3. Ca^{2+} -dependent release of ^{125}I -CaM from E95 and E105

A blot of ciliary protein from a 7% gel was cut into strips, incubated with ^{125}I -CaM (0.5 nM) in buffer HE (1 mM-HEDTA) and washed with four changes of buffer HE. The strips then were washed in buffer HE with various amounts of calcium acetate (lanes 1–8) or magnesium acetate (lanes 9–12) for 1 h. Final wash solutions contained 200 nM-bovine CaM to block re-association of ^{125}I -CaM with the Ca^{2+} -dependent CaM-binding proteins. The free Ca^{2+} concentration was: lane 1, 50 nM; lane 2, 100 nM; lane 3, 300 nM; lane 4, 1.4 μM ; lane 5, 4.0 μM ; lane 6, 7.0 μM ; lane 7, 10 μM ; lane 8, 40 μM . The free Mg^{2+} concentration was: lane 9, 10 μM ; lane 10, 100 μM ; lane 11, 200 μM ; lane 12, 1 mM. Each lane contained approx. 70 μg of ciliary protein. In separate experiments, Ca^{2+} -dependent release of ^{125}I -CaM was also observed in the absence of unlabelled bovine CaM.

Fig. 4. Competitive displacement of ^{125}I -CaM binding to the Ca^{2+} -dependent CaM-binding proteins

A blot of ciliary proteins from a 7% gel was cut into strips and incubated in 1 mM- CaCl_2 (buffer C) with ^{125}I -CaM (0.4 nM) and unlabelled *Paramecium* CaM at 1 nM (lane 2), 2 nM (lane 3), 5 nM (lane 4), 10 nM (lane 5), 20 nM (lane 6), and 50 nM (lane 7); unlabelled bovine CaM at 2.5 nM (lane 9), 5 nM (lane 10), 10 nM (lane 11), 25 nM (lane 12) and 50 nM (lane 13); or troponin C at 25 nM (lane 14), 50 nM (lane 15), 100 nM (lane 16) and 500 nM (lane 17). Lanes 1 and 8 were control strips incubated in ^{125}I -CaM without any added competitor. Each lane contained approx. 50 μg of ciliary protein.

and one that is Ca^{2+} -dependent. For convenience we will call this protein 'E105' with reference to the Ca^{2+} -inhibitable binding sites suggested by Fig. 3.

Specificity of binding

To determine whether the Ca^{2+} -dependent CaM-binding proteins bound CaM specifically, blots of whole cilia were incubated with ^{125}I -CaM and various amounts of unlabelled *Paramecium* CaM, bovine CaM or troponin C. Both bovine CaM and *Paramecium* CaM displaced the labelled probe at low concentrations of unlabelled competitor, although *Paramecium* CaM was slightly more effective for some proteins (Fig. 4). Although the conditions of the assay clearly do not permit estimation of true affinity constants, the half-maximal inhibitory concentrations determined by densitometry (1–25 nM) suggested high-affinity interactions of these proteins with CaM similar to those reported for several known CaM-activated enzymes [50]. By contrast, troponin C, a Ca^{2+} -binding protein related in sequence and structure to CaM [51], was a relatively weak competitor of the labelling of C63, C126, and C36. The labelling of the 75, 85 and 96 kDa proteins, however, was more effectively inhibited by troponin C, with a half-maximal inhibitory concentration equal to, or slightly higher than, that of *Paramecium* CaM (see Fig. 4). Parvalbumin, a more distantly related Ca^{2+} -binding protein, did not displace the label from any of these polypeptides (results not shown). Therefore the binding of CaM to the major CaM-binding activities is specific for CaM over closely related proteins, whereas some of the minor binding

activities may have a more general affinity for CaM-related proteins.

Competitive-displacement experiments were also performed to determine the specificity of interaction between CaM and the Ca^{2+} -inhibitable CaM-binding proteins. As summarized in Table 1, unlabelled *Paramecium* CaM competed effectively with the labelled CaM for binding to E95 and E105 at low concentrations. In contrast with the Ca^{2+} -dependent CaM-binding proteins, however, bovine CaM was a very poor com-

Table 1. Competitive displacement of ^{125}I -CaM binding to E95 and E105 by unlabelled CaMs and related Ca^{2+} -binding proteins

Blots of whole cilia were incubated in Buffer E with ^{125}I -CaM (0.5 nM) and various concentrations of unlabelled *Paramecium* CaM, bovine CaM, troponin C, or parvalbumin. The IC_{50} values (mean $\pm$ S.D. for n experiments) represent estimates of competitor concentrations giving half-maximal displacement of ^{125}I -CaM, and were determined by densitometric scans of the autoradiographs. The data for both E95 and E105 were similar and are combined.

Competitor	IC_{50} (nM)	n
<i>Paramecium</i> CaM	19 ± 8	4
Bovine CaM	> 150	5
Troponin C	> 200	3
Parvalbumin	> 200	2

Fig. 5. Subciliary distribution of CaM-binding proteins

Freshly prepared cilia were demembrated with Triton X-100. Intact cilia and the extracted cilia were sedimented separately through discontinuous sucrose gradients and the resulting three layers of material were collected from each gradient. Aliquots representing 10% of total protein from each fraction were analysed by SDS/PAGE (6–10% gradient gel) and ^{125}I -CaM blot. (a) Shows the Coomassie Blue-stained gel. Lane 1 contains whole cilia (100 μg); lane 2, membrane and matrix (50 μg); lane 3, axonemes (25 μg). Lanes 4, 5 and 6 contain proteins from the CMV, IDC and axoneme layers respectively from the sucrose gradient of intact cilia (see the Experimental section). Lanes 7, 8 and 9 contain CMV, IDC, and axoneme layers from the gradient of detergent-extracted cilia. (b) Shows the autoradiograph after ^{125}I -CaM blot with 1 mM- CaCl_2 . The samples in each lane are the same as in (a), except that lane

petitor. Troponin C and parvalbumin failed to inhibit the labelling of these polypeptides. Thus E95 and E105 show remarkable specificity for *Paramecium* CaM, suggesting a more stringent structural requirement for successful interaction. That these proteins can bind unlabelled CaM further shows that binding to these polypeptides is not a result of the iodination procedure.

Inhibition of binding by melittin

The bee-venom peptide melittin has been shown to be a potent inhibitor of the CaM-dependent enzymes PDE and MLCK [52,53]. Like other CaM-antagonists, melittin inhibits competitively through a Ca^{2+} -dependent association with CaM [53]. Melittin also blocks activation of brain PDE and MLCK by *Paramecium* CaM (R. D. Hinrichsen, unpublished work). Similarly, we found that melittin completely blocked the binding of ^{125}I -CaM to all of the Ca^{2+} -dependent CaM-binding proteins of whole cilia, with 50% inhibition occurring between 50 and 200 nM-melittin (results not shown). The binding activities of E95 and E105, however, were not affected by this peptide (results not shown).

Subciliary distribution

Like other cilia and flagella, *Paramecium* cilia consist of an axoneme core enclosed by a membrane that is continuous with the cell-body membrane. Previous work has shown that *Paramecium* cilia can be demembrated either by non-ionic detergents, yielding soluble 'membrane-matrix' and insoluble 'axoneme' fractions, or by hypo-osmotic shock, which yields a mixture of ciliary membrane vesicles (CMV), incompletely demembrated cilia (IDC) and demembrated axonemes [38]. These components can be separated by sedimentation of the extracts on discontinuous sucrose gradients [38]. To determine the subciliary distribution of the ciliary CaM-binding proteins, we assayed demembration extracts and sucrose-gradient fractions with the ^{125}I -CaM blot method.

Extraction of cilia with Triton X-100 solubilized most of the I-antigen whereas the dynein heavy chains and tubulin remained with the axoneme pellet (Fig. 5a, lanes 1–3). Most of the CaM-binding activity, including C63, C126, E95 and E105, was associated with the pellet (Figs. 5b and 5c, lane 3). Prolonged extraction (up to 1.5 h) failed to remove these proteins. However, extraction of axonemes with high-ionic-strength buffers (0.6 M-NaCl or -KI) solubilized a significant fraction (50–80%) of these CaM-binding proteins together with a large proportion of the dynein heavy-chain polypeptides (results not shown). Similar methods are used routinely to solubilize axonemal components of other organisms [54,55]. These results suggest that these CaM-binding proteins are associated with the axoneme. By contrast, C36 was solubilized from cilia by detergent (Fig. 5b, lane 2) and also was largely solubilized by the hypo-osmotic-

3 contains 50 μg of axoneme protein instead of 25 μg . (c) Shows the autoradiograph after ^{125}I -CaM blot in 1 mM-EGTA. Only the region containing E95 and E105 is shown. The lanes contained the same samples as in (b). The samples in lanes 1–3 and lanes 4–9 were from separate experiments. The position of the CaM-binding proteins, dynein heavy chains (D), tubulins (T) and I-antigen (I) are noted. The molecular-mass standards (S) in (a) are described in the Experimental section.

shock method (results not shown). Note that a protein of similar size was also detected in the deciliation supernatant (Fig. 1*b*, lane 3). Thus C36 may be a soluble protein or it may be easily dislodged from ciliary structures during fractionation.

Sucrose-density-gradient fractionation resolved both 'intact' cilia and demembrated cilia into three distinct layers of material. When intact cilia were resolved, most of the tubulin and dynein heavy chains sedimented to the IDC layer (see the Experimental section; Fig. 5*a*, lane 5). Some I-antigen travelled to the CMV layer (Fig. 5*a*, lane 4), and low-molecular-mass proteins, together with some tubulin and dynein, banded at the axoneme layer, indicating some degree of demembration. Complete demembration of cilia by Triton treatment resulted in a shift in the distribution of the axonemal proteins, with most of these proteins sedimenting with the axoneme layer (Fig. 5*a*, lanes 7–9). Similarly, resolution of hypo-osmotic-shock extracts on sucrose gradients revealed a redistribution of tubulin and dynein polypeptides between the IDC and axoneme layers (results not shown). Examination of the CaM-binding proteins in these fractions revealed that C63, C126 and C70–100 always co-sedimented with tubulin and dynein before and after membrane extraction (Fig. 5*b*). Similarly, E95 and E105 also redistributed with the axonemal proteins after demembration (Fig. 5*c*). These results further support the localization of these CaM-binding proteins to the axoneme.

We also tested whether any CaM-binding proteins may have been contributed by trichocysts, which are rod-like structures secreted from cells during cell harvest.

Fig. 6. Comparison of the CaM-binding protein pattern of CMVs and Triton X-100 extracts of cilia

CMVs were prepared by hypo-osmotic shock of cilia and were isolated from the CMV layer of a discontinuous sucrose gradient loaded with this extract. A 50 µg portion of CMV protein (lane 1) and 100 µg of the Triton X-100 extract of cilia (lane 2) were run on a 6–10% acrylamide gradient gel. The autoradiograph after ^{125}I -CaM blot in 1 mM- CaCl_2 (buffer C) is shown.

Trichocysts are minor contaminants in most ciliary preparations (0.1–5% of total protein; results not shown). Two lines of evidence suggest that the CaM-binding proteins were not derived from trichocysts: (1) purified trichocysts contained no detectable CaM-binding proteins (results not shown); (2) trichocyst proteins did not redistribute in sucrose gradients after demembration, but banded predominantly in the axoneme layer with or without demembration, and thus did not correlate with the CaM-binding-protein pattern of fractionation. In Fig. 5*(a)* the proteins between the 14 and 21 kDa markers are predominantly trichocyst proteins [38,56].

Given the importance of the ciliary membrane to control of motility and the number of Ca^{2+} -dependent membrane functions, we examined more closely the CaM-binding activities of ciliary membranes. Although C36 was the most prominent CaM-binding activity in the membrane-matrix fraction, longer exposures of the autoradiograms revealed additional CaM-binding proteins, including bands at 55, 63, 70 and 120 kDa (Fig. 6). Purified CMVs also contained CaM-binding polypeptides of 63, 70 and 120 kDa, as well as a faintly labelled band at 36 kDa (Fig. 6). The relative binding activity of the 36 kDa protein was variable between separate CMV preparations. All of these polypeptides bound ^{125}I -CaM Ca^{2+} -dependently and the labelling was blocked by excess unlabelled CaM (results not shown). Both *Paramecium* CaM and bovine CaM were effective competitors (50% inhibition at < 25 nM), but troponin C competed weakly (results not shown). Therefore, additional CaM targets may be associated with the ciliary membrane.

Binding of ^{125}I -CaM to intact axonemes

The observed pattern of CaM-binding polypeptides in axonemal fractions predicts that axonemes contain both

Fig. 7. Dependence on CaM concentration of ^{125}I -CaM binding to intact axonemes

Increasing concentrations of ^{125}I -CaM at a constant specific activity were incubated with isolated intact axonemes, and the amount bound was determined as in the Experimental section. Axonemes were incubated with CaM at a free Ca^{2+} concentration of 70 µM (●) or 0.02 µM (○). All values were corrected for 'non-saturable' binding measured in the presence of 1.5 µM-unlabelled CaM.

Fig. 8. Competitive displacement of ^{125}I -CaM binding to axonemes by unlabelled *Paramecium* CaM, bovine CaM and troponin C in high Ca^{2+} and low Ca^{2+} .

Various amounts of unlabelled *Paramecium* CaM (●), bovine CaM (○) or troponin C (×) were mixed with 1 nM- ^{125}I -CaM and then incubated with axonemes at free Ca^{2+} concentrations of 70 μM (a) or 0.02 μM (b) for 60 min. The percentage of radioactivity bound, relative to the radioactivity bound in the absence of unlabelled competitor, was determined and plotted against the concentration of unlabelled competitor. The amount of ^{125}I -CaM bound in the absence of unlabelled competitor was 173 ± 4 fmol/mg at 70 μM - Ca^{2+} and 184 ± 13 fmol/mg at 0.02 μM - Ca^{2+} .

Ca^{2+} -dependent and Ca^{2+} -inhibitable binding sites for CaM. Therefore we assayed for the binding of ^{125}I -CaM to isolated intact axonemes using a filtration assay. At both low (20 nM) and high (70 μM) free Ca^{2+} concentrations, saturable binding of the labelled CaM was observed (Fig. 7). Surprisingly, the extent of binding was largely independent of Ca^{2+} . The maximal extent of binding suggested at least 5–10 pmol of available binding sites per mg of axoneme at both low and high Ca^{2+} . The

total amount of endogenous CaM in the axonemal preparations, as estimated by immunoblot analysis (see the Experimental section), was less than 5 pmol/mg, suggesting that the number of CaM binding sites masked by endogenous CaM was small (results not shown). Partially purified trichocysts, at protein concentrations equal to the maximum found in axoneme samples, bound only minimal amounts of CaM (< 10 % of the binding of axoneme samples at 1 nM- ^{125}I -CaM; results not shown). Therefore trichocysts did not contribute significantly to the CaM-binding activities of axonemes under our assay conditions.

In both the presence and absence of Ca^{2+} , more than 90 % of the binding was specific (at 1 nM- ^{125}I -CaM) on the basis of displacement of label by excess unlabelled *Paramecium* CaM (Figs. 8a and 8b). The estimated concentrations of *Paramecium* CaM giving 50 % inhibition (IC_{50}) were between 3 and 10 nM at both 70 μM - and 0.02 μM - Ca^{2+} , suggesting binding sites of high affinity. However, comparison of the potencies of *Paramecium* CaM, bovine CaM and troponin C in displacing ^{125}I -CaM revealed differences in the binding at low compared with high Ca^{2+} concentrations. At 70 μM - Ca^{2+} , unlabelled *Paramecium* CaM was slightly more effective than bovine CaM (IC_{50} approx. 15–22 nM), and significantly more effective than troponin C, although troponin C did compete ($\text{IC}_{50} \approx 110$ nM) (Fig. 8a). By contrast, the binding to axonemes at 0.02 μM - Ca^{2+} was weakly displaced by unlabelled bovine CaM ($\text{IC}_{50} \approx 150$ nM), and troponin C was ineffective at concentrations up to 400 nM (Fig. 8b). In addition, we found that melittin potentially inhibited binding in high Ca^{2+} , but was less effective in low Ca^{2+} (results not shown). These results suggested that at least some of the binding sites detected in high- Ca^{2+} buffers were distinct from those observed in low- Ca^{2+} buffers, implying the existence of both Ca^{2+} -dependent and Ca^{2+} -inhibitable binding sites.

To further distinguish these binding sites and to test their Ca^{2+} -dependence, axonemes were labelled with ^{125}I -CaM in low or high Ca^{2+} , then diluted into solutions of different Ca^{2+} concentrations, and the amount of ^{125}I -CaM remaining bound was determined. As Table 2

Table 2. Release of ^{125}I -CaM from prelabelled axonemes

Axonemes were labelled for 1 h with ^{125}I -CaM (1 nM) in high (50 μM) or low (0.02 μM) Ca^{2+} . Samples from each tube then were diluted 50-fold into high- or low- Ca^{2+} buffer (wash) for 45 min, and the amount of ^{125}I -CaM remaining bound was determined. In some washes, 250 nM-bovine CaM (B-CaM) was included. Three samples were assayed for each condition and the values represent the mean $\pm$ S.D.

Incubation	Wash	^{125}I -CaM bound (fmol/mg)
High Ca^{2+}	High Ca^{2+}	65 ± 4
	Low Ca^{2+}	56 ± 2
Low Ca^{2+}	High Ca^{2+}	38 ± 2
	Low Ca^{2+}	73 ± 4
	High Ca^{2+}	22 ± 3
	+ B-CaM	
	Low Ca^{2+}	67 ± 7
	+ B-CaM	

shows, CaM bound in low Ca^{2+} was more efficiently released if Ca^{2+} concentrations were raised to $50 \mu\text{M}$ in the wash. When excess bovine CaM was included in the wash buffers, the removal of ^{125}I -CaM by Ca^{2+} was significantly enhanced. This result suggests that bovine CaM may prevent exchange of ^{125}I -CaM from low- Ca^{2+} sites to high- Ca^{2+} sites when the Ca^{2+} concentration is raised. These results support the presence of Ca^{2+} -inhibitable CaM targets in axonemes. By contrast, the removal of ^{125}I -CaM bound to axonemes in high Ca^{2+} was not consistently affected by the Ca^{2+} concentration in the wash (see the Discussion section).

DISCUSSION

Although several investigators have provided intriguing data to suggest roles for CaM in the regulation of ciliary and flagellar motion, little is known of its protein targets or the biochemical pathways that they regulate. The results presented here suggest the presence of at least two types of CaM-binding proteins in *Paramecium* cilia. One group exhibits properties typical of many known CaM-regulated enzymes and proteins [49]; they bind CaM Ca^{2+} -dependently, and are inhibited by the CaM antagonist melittin. A second type of protein is unusual in that it binds CaM with high affinity only when Ca^{2+} concentrations are below the micromolar range. One of these polypeptides, E105, may also contain Ca^{2+} -dependent binding sites for CaM.

Competitive-displacement experiments demonstrated that the major CaM-binding activities were of high affinity and specificity; they bound *Paramecium* CaM at nanomolar levels, but bound closely related Ca^{2+} -binding proteins less effectively. In particular, E95 and E105 showed remarkable specificity for *Paramecium* CaM over bovine CaM. *Paramecium* CaM differs from bovine CaM at only 19 amino acid residues, although it also contains a novel dimethyl-lysine residue [32]. Moreover, like bovine CaM, *Paramecium* CaM activates vertebrate brain PDE and muscle MLCK [18,31]. Therefore, the specificity of the binding interactions strongly argues against association of CaM with these proteins due simply to its general acidic or hydrophobic properties.

Ciliary subfractionation experiments suggested that the ciliary targets for CaM include soluble, membrane-bound and axonemal proteins. The Ca^{2+} -inhibitable targets and many of the Ca^{2+} -dependent CaM-binding proteins were associated with axonemes. Similarly, filtration binding experiments revealed specific and high-affinity binding sites for CaM in preparations of intact axonemes. Although the extent of binding was Ca^{2+} -independent, the relative potencies of bovine CaM and troponin C in displacing ^{125}I -CaM was Ca^{2+} -dependent, indicating that these axonemal binding sites are heterogeneous. Furthermore, CaM that bound to axonemes in low Ca^{2+} could be partially removed by raising the Ca^{2+} concentration, suggesting the presence of Ca^{2+} -inhibitable binding sites. The removal of ^{125}I -CaM from 'high- Ca^{2+} ' sites, however, was not consistently affected by Ca^{2+} . It may be that efficient exchange of CaM between Ca^{2+} -dependent and Ca^{2+} -inhibitable sites obscures measurements of the reversibility of binding. Indeed bovine CaM, which has weak affinity for the Ca^{2+} -inhibitable CaM-binding proteins, enhanced the removal of CaM from the 'low- Ca^{2+} ' sites by Ca^{2+} , presumably by selectively blocking reassociation of ^{125}I -

CaM with 'high- Ca^{2+} ' sites. In any case, these results, together with the ^{125}I -CaM blot data, are consistent with the presence of both Ca^{2+} -dependent and Ca^{2+} -inhibitable CaM targets in axonemes. Like E95 and E105, the Ca^{2+} -inhibitable binding sites on intact axonemes showed strong specificity for *Paramecium* CaM over bovine CaM. In addition, melittin inhibited the binding of CaM to intact axonemes in high Ca^{2+} , a property shared with the Ca^{2+} -dependent CaM-binding proteins.

It should be noted that the conditions inherent to the ^{125}I -CaM blot assay may have prevented the detection of some CaM targets. Furthermore, the distribution of label on protein blots may not accurately reflect the true relative abundance of CaM-binding proteins in the cilium. It also is conceivable that some proteins that bind ^{125}I -CaM *in vitro* actually are targets for a modified form of CaM or a distinct CaM-like protein *in vivo*. Suggestive data for distinct CaM-like proteins or modified CaM in the flagella of *Chlamydomonas* [57] and *Naegleria* [58], and in *Tetrahymena* cilia [59], have been reported.

Considered together, these results imply that CaM has multiple targets in the *Paramecium* cilium, and, hence, possibly plays several functional roles. They further suggest the intriguing possibility that CaM may activate distinct sets of proteins as Ca^{2+} concentrations fluctuate during and after membrane excitation (see Fig. 2). Therefore, CaM may translate different changes in Ca^{2+} into qualitatively distinct physiological responses. Data from other organisms also are consistent with complexity in the CaM-dependent regulation of ciliary motility. Both dissociable and non-dissociable pools of CaM were detected in *Chlamydomonas* [25] and sperm [28] flagella. Numerous potential CaM targets have also been described in *Tetrahymena* cilia [60], including several detergent-insoluble Ca^{2+} -dependent proteins of similar M_r to those reported here. However, Ca^{2+} -inhibitable CaM-binding proteins were not detected, probably due to differences from our assay conditions (T. C. Evans, unpublished work). In *Mytilus* (mussel) gill cilia, Ca^{2+} -independent and Ca^{2+} -dependent CaM-binding proteins, in addition to a 13 kDa Ca^{2+} -inhibitable protein, were observed [61]. The CaM-specificity and Ca^{2+} -sensitivity of these proteins is not known.

What functions might these CaM-binding proteins regulate?

One or more of the CaM targets may regulate, directly or indirectly, the axonemal component(s) that control ciliary-beat direction. Recent studies indicate that the Ca^{2+} -mediated ciliary reversal pathway and the regulation of forward swimming by cyclic nucleotides are closely interrelated; cyclic AMP and cyclic GMP inhibit Ca^{2+} -stimulated ciliary reversal in permeabilized cells [8,10], and Ca^{2+} regulates cyclic AMP and cyclic GMP synthesis *in vitro* [12,13] and cyclic GMP *in vivo* [11]. Therefore CaM may modulate ciliary reversal indirectly by influencing cyclic nucleotide effects on the axoneme, a possibility supported by the ability of CaM antagonists to augment the inhibition of ciliary reversal by cyclic AMP [21]. In addition, a Ca^{2+} - and CaM-stimulatable guanylate cyclase activity is present in the ciliary membrane [13].

Ca^{2+} may also regulate ciliary-beat frequency both in the forward [15]- and backward [14]-beating modes. Thus dyneins, the enzymes that provide motive force for

the cilium, are potential targets for Ca^{2+} and CaM control. In *Tetrahymena* [62] and in sea-urchin [63], Ca^{2+} - and CaM-dependent activation of partially purified dynein ATPase has been shown. Although a Ca^{2+} -sensitive ATPase has also been found in *Paramecium* cilia, calmodulin antagonists failed to inhibit this activity, and endogenous CaM was not associated with 22 S or 12 S dyneins [64]. Preliminary experiments revealed that the CaM-binding proteins extracted from axonemes did not co-sediment with 22 S or 12 S dynein polypeptides on linear sucrose gradients (T. C. Evans, unpublished work). Thus whether the dyneins of *Paramecium* are regulated by CaM or perhaps a different Ca^{2+} -binding protein demands further investigation.

Several Ca^{2+} -dependent ion conductances are crucial to the regulation of Ca^{2+} flux into cilia [7]. Interestingly, the loss of the Ca^{2+} -dependent K^+ current in the *pntA* mutant results from a single amino acid substitution in CaM [23]. In preliminary experiments, CaM from *pntA* bound poorly to the C36 polypeptide compared with wild-type CaM (both in CMV and in detergent extracts), whereas interactions with other ciliary membrane/matrix CaM-binding proteins were not detectably altered (R. D. Hinrichsen, A. Burgess-Cassler, T. C. Evans & C. Kung, unpublished work). C36, therefore, may be involved in the regulation of this membrane conductance. The presence of a C36-like protein in CMV suggests that C36 molecules may associate with the ciliary membrane or they may become trapped within sealed vesicles. Although the phenotype of *pntA* is relatively specific (only the Ca^{2+} -dependent K^+ currents and mating are substantially altered [65]), *pntA* CaM activates PDE and MLCK to a similar extent as wild-type CaM [31]. Thus the *pntA* mutation may subtly alter some Ca^{2+} - and CaM-regulated processes while leaving others unaffected. Indeed, considering the many potential roles for CaM in cells [49], any severe defect may prove lethal, an idea supported by genetic studies of CaM function in yeast [66].

The Ca^{2+} -inhibitable CaM-binding proteins E95 and E105 are particularly intriguing, as they may represent a newly emerging class of proteins of which little is known. Similar proteins have been reported in other organisms [67,68]. Storm and colleagues [69–71] have extensively studied a membrane protein in the mammalian nervous system that also binds CaM best at submicromolar Ca^{2+} concentrations. Because of its high concentrations, it may sequester and localize CaM when Ca^{2+} concentrations are low. Either E95 or E105 could have a similar function in cilia, perhaps localizing CaM to specific sites on the axoneme. Alternatively, one or both of these polypeptides may act catalytically, perhaps in an antagonistic manner to a Ca^{2+} -dependent activity. Such a system could provide tight on/off control of a Ca^{2+} -stimulated pathway, particularly if the Ca^{2+} -inhibitable and Ca^{2+} -dependent activities were closely juxtaposed on the axoneme.

Future studies of the *Paramecium* CaM-binding proteins will need to focus on assessment of their roles in regulating motility and on identification of their biochemical functions and localization in the cilium. The feasibility of genetic and biochemical analyses together with behavioural assays accessible to macromolecules [10,29] offer promise of addressing these questions in *Paramecium* in the near future. Moreover, the ciliary system of *Paramecium* provides an excellent model for

studying the mechanisms and interrelationships of CaM-dependent pathways. Given the ubiquity of CaM in nature, this is an endeavour of broad relevance.

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