# Presence and Indirect Immunofluorescent Localization of Calmodulin in *Paramecium tetraurelia*

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ABSTRACT In this paper we demonstrate the presence and localization of calmodulin, a calcium-dependent regulatory protein, in the ciliated protozoan *Paramecium tetraurelia.* Calmodulin is demonstrated by several criteria: (*a*) the ability of whole cell *Paramecium* extracts to stimulate mammalian phosphodiesterase activity, (*b*) the presence of an acidic, thermostable, 17,000-dalton polypeptide whose mobility shifts in SDS polyacrylamide gel electrophoresis in the presence of $Ca^{2+}$, and (*c*) the affinity of antibodies against mammalian calmodulin for a *Paramecium* component as demonstrated by both indirect immunofluorescent localization and radioimmunoassay. Indirect immunofluorescence studies reveal that *Paramecium* calmodulin is distributed in three distinct regions of the cell, i.e., (*a*) large, spherical cytoplasmic organelles representing perhaps the food vacuoles or other vacuolar inclusions of the cell, (*b*) along the entire length of oral and somatic cilia, and (*c*) along a linear punctate pattern corresponding to the kineties (basal bodies) of the cell.

Calmodulin, a calcium-dependent regulatory protein, was originally identified as an activator of 3',5'-cyclic AMP phosphodiesterase (PDE) activity (5, 16), and has since been shown to be an intermediate in a large number of intracellular calcium-regulated processes (6, 26, 37), suggesting that it may be of fundamental importance in mechanisms by which calcium ions regulate events within cells. It is a ubiquitous, acidic, thermostable protein with a native molecular weight of ~17,000 and possesses a highly conserved structure containing four divalent cation-binding sites (10).

In this report we present evidence for the presence and localization of calmodulin in the ciliated protozoan *Paramecium tetraurelia.* The presence of calmodulin and its distribution in the ciliates is of special interest because these cells are studied as model systems for a number of calcium-regulated processes. An increase in cytoplasmic calcium ion concentration has been shown to be important for both stimulus-secretion coupling (33) and for regulation of ciliary beat direction in *Paramecium* (28). A preliminary report of these findings was presented at the Society of Protozoology Meetings, August, 1979 (23).

## MATERIALS AND METHODS

### Cultural Growth Conditions

*Paramecium tetraurelia* (wt) is grown in either axenic (modified after Soldo et al. [35]), or monoaxenic culture of *Enterobacter aerogenes* in Cerophyl medium at 27°C. Cells are harvested during early stationary phase (9,000 cells per ml axenic; 3,500 cells per ml monoaxenic) by centrifugation at 1,000 rpm for 1 min at room temperature in an IEC HN SII centrifuge (Damon/IEC Div., Damon Corp., Needham Heights, Mass.).

### PDE Stimulation Assay

Harvested cells are washed once in Dryl's solution (13), homogenized on ice in a Dounce 15-ml, tight-fitting homogenizer (Kontes Co., Vineland, N. J.) for 1 min. The homogenate is then assayed after heat treatment, following the procedure outlined in reference 9.

### Preparation of Purified Bovine Brain Calmodulin

Calmodulin was purified from bovine brain by ammonium sulfate precipitation and ion exchange chromatography using a protocol modified after Watterson and co-workers (38).

### Preparation of Crude Calmodulin Extract for Gel Electrophoresis

Cells are homogenized at 4°C in a buffer consisting of 20 mM Tris, 1 mM NaCl, 0.04% Traysylol and 0.1% soybean trypsin inhibitor, pH 6.5, using a Dounce homogenizer. The homogenate is centrifuged at 20,000 *g* for 30 min. The supernate is collected and the pellet reextracted as described above. The supernates are combined and centrifuged again at 75,000 *g* for 1 h. The resulting supernate is heated for 6 min at 85°C in a water bath and then immediately immersed in ice and subsequently spun at 20,000 *g* for 30 min. This supernate is made 35% ammonium sulfate (wt/vol; pH 7.0) on ice for 2 h, spun at 20,000 *g* for 1 h, and the supernate is collected. The supernate is made 50% ammonium sulfate (wt/vol; pH 4.0) on ice for 2 h, spun at 20,000 *g* for 1 h, and the pellet is collected. The pellet is resuspended in buffer and dialyzed for 12 h against 2l of 20 mM imidazole and 20 mM NaCl buffer at pH 7.0. Samples are treated with an equal

volume of standard twofold SDS buffer for polyacrylamide gel electrophoresis (PAGE) analysis and run in 15% polyacrylamide slab gels containing 0.1% SDS (19) in the presence of either 5.0 mM $CaCl_2$ or 1.0 mM EGTA.

## Radioimmunoassay

*Paramecium* cells were homogenized and heated at 90°C for 2 min followed by quick-cooling in a dry ice–methanol bath. After centrifugation for 30 sec in a Beckman microfuge, calmodulin was quantitated in the supernates by the radioimmunoassay procedure described by Chafouleas et al. (4).

## Indirect Immunofluorescent Localization

Cells are fixed in 3% formalin for 30 min, postfixed in −20°C absolute acetone for 20 min, and preincubated in 0.01% bovine serum albumin (BSA) before incubation with the primary antibody, goat anticalmodulin. This antibody was prepared using antigen isolated from rat testes, purified by affinity chromatography, and characterized by Dedman and co-workers (11).

Cells are incubated in antibody in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, for 30 min at 37°C. After two 30-min washes in buffer the cells are again preincubated in 0.01% BSA, then incubated in a × 300 dilution of rabbit anti-goat rhodamine conjugated IgG (N. L. Cappel Laboratories Inc., Cochranville, Pa.). All washes between treatments were carried out in PBS. Three different controls were performed to insure the immunospecificity of the results including: (*a*) incubation of cells in secondary antibody without prior incubation in primary antibody, (*b*) incubation of cells in the nonadsorbed fraction during affinity chromatographic purification of the antibody, and (*c*) incubation of cells in primary antibody which itself has been preincubated in a 6-M excess of purified brain calmodulin standard (Fig. 1 *B*, lane *3*) overnight at 4°C.

Cells are examined in a Zeiss Universal epi-illuminated fluorescence microscope. Light micrographs are obtained on Tri-X film (Kodak; developed in Diafine, 5 min). All experimental micrographs represent 2-min fluorescence exposure; comparable control images represent 4-min film exposure.

## RESULTS

PDE[1] catalyzes a reaction in which cAMP is hydrolyzed to 5′-AMP. Calmodulin activates one form of this enzyme in the presence of physiological concentrations of calcium. Results of the PDE stimulation assay indicate that calmodulin is present in heat-treated whole-cell homogenates (Fig. 1 *A*). As illustrated here, the *Paramecium* extract under these conditions stimulates PDE activity to the same extent as both rat testes calmodulin and rat testes extract. Binding of phenothiazine derivatives to calmodulin has been shown to inhibit PDE stimulation by this activator (21, 22). One such derivative, trifluoperazine (SmithKline Corp., Philadelphia, Pa.), has been used as an effective probe for the presence of calmodulin in *Paramecium*. Under separate assay conditions, 18 μM trifluoperazine added to the reaction mixture reduces PDE stimulation by *Paramecium* extracts from 50 ng/mg per min to 0 ng/mg per min. This complete inhibition of PDE stimulation by *Paramecium* whole-cell extracts suggests that *Paramecium* calmodulin is also capable of binding trifluoperazine. In addition, further evidence for the presence of calmodulin in *Paramecium* which is antigenically similar to mammalian calmodulin is demonstrated by radioimmunoassay (Fig. 1 *C*). This figure demonstrates that both heat-treated extracts of rat testes and *Paramecium* exhibit similar competitive binding properties under standard radioimmunoassay conditions.

The partially purified calmodulin extract was run on a 15% polyacrylamide gel containing 0.1% SDS in the presence of either 5 mM $CaCl_2$ or 1 mM EGTA (Fig. 1 *B*). In the presence of 1 mM EGTA, a polypeptide migrates with an apparent molecular mass of ~17,000, slightly faster than the bovine brain calmodulin standard, which exhibits an apparent molecular mass of 18,500. The relative shift in mobility of this polypeptide (arrows) when the electrophoretic sample is made

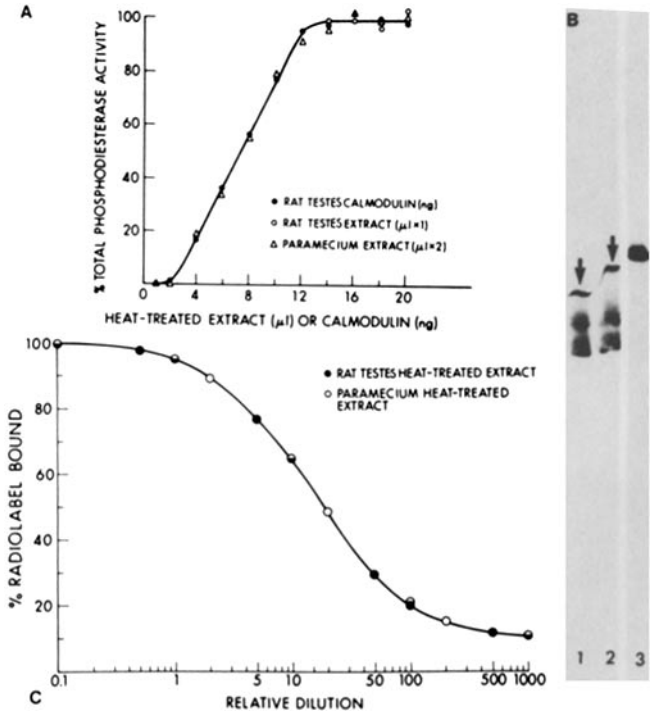


FIGURE 1 (*A*) Results of PDE assay. Stimulation of PDE by rat testes calmodulin, rat testes, and *Paramecium* heat-treated extracts. (*B*) 15% Polyacrylamide slab gel electrophoresis. Protein from *Paramecium* crude calmodulin extract is loaded on lanes *1* and *2*. Lane *3*, standard bovine brain calmodulin in presence of 5.0 mM $CaCl_2$. Lane *1* represents crude *Paramecium* calmodulin extract in the presence of 5.0 mM $CaCl_2$. Lane *2* is loaded with crude calmodulin extract in the presence of 1.0 mM EGTA. The characteristic mobility shift (arrows) of *Paramecium* calmodulin in the presence or absence of $CaCl_2$ is clearly illustrated here. (*C*) Radioimmunoassay. Different dilutions of *Paramecium* and rat testes heat-treated extracts show the same competitive binding by anticalmodulin.

5 mM $CaCl_2$ provides further evidence that this polypeptide is calmodulin, since other calcium-binding proteins do not undergo this mobility shift in the presence of calcium in SDS PAGE (3).

Calmodulin localization by indirect immunofluorescence is illustrated in Figs. 2 and 3. Fig. 2 *A* is a phase-contrast image of a control cell. Fig. 2 *B* is a representative control image in which the cell has not been incubated with primary antibody but has been exposed to the secondary label. In these cells, as well as in all control experiments performed, only a diffuse low level of background fluorescence is visible. Where cells are treated with anticalmodulin followed by fluorescent-labeled secondary antibody, fluorescence is seen localized in three distinct regions of the cell: (*a*) Label is present in uniformly sized (15 μm diameter) vacuolar inclusions (Fig. 2 *C*). These labeled organelles vary in number within each cell, ranging from 0 to 5, and are present in both axenically and monoaxenically cultured *Paramecium* but are absent in *Tetrahymena* (24, 32). (*b*) Anticalmodulin localizes along the entire length of both oral and somatic cilia (Fig. 2 *D*). (*c*) Label is also present in deciliated cells in a linear punctate array of fluorescent spots (Fig. 3 *A* and *B*). This last pattern of localization corresponds to the location of the basal bodies.

## DISCUSSION

Calmodulin is known as an activator of a growing list of enzymes including PDE (5, 16), adenylate cyclase (2, 7), myosin

[1] PDE: 3′5′-cyclic nucleotide hydrolase (EC 3.1.4.17).

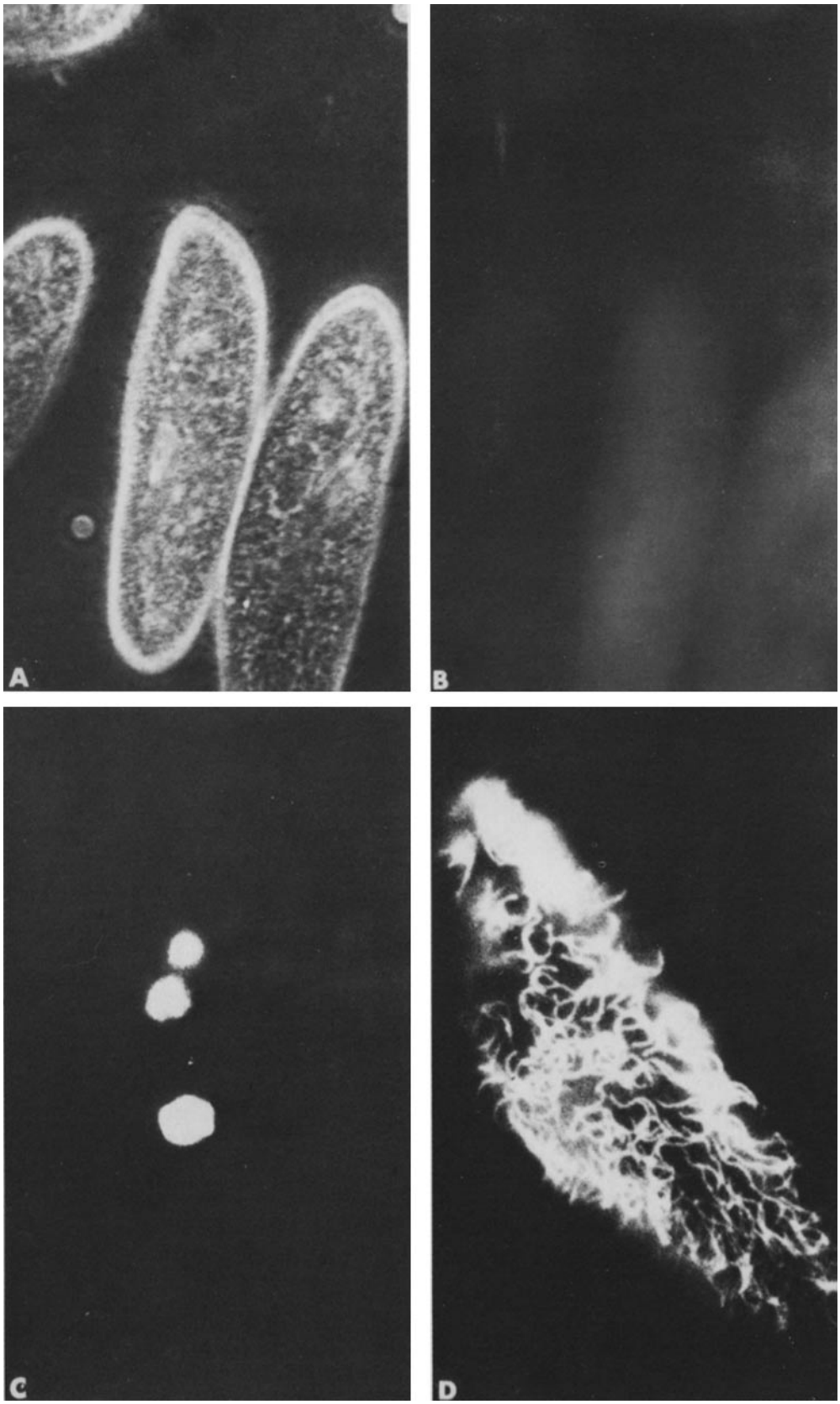


FIGURE 2 Immunofluorescence microscopy in *Paramecium*. Fig. 2 *A* is a light micrograph of the immunofluorescent control cells shown in Fig. 2 *B*. These control cells have been exposed only to the secondary label and not been incubated in primary antibody. Fig. 2 *C* illustrates anticalmodulin staining fluorescent vacuolar inclusions (15 μm) and Fig. 2 *D* anticalmodulin staining cilia that fluoresce along their entire length. × 256.

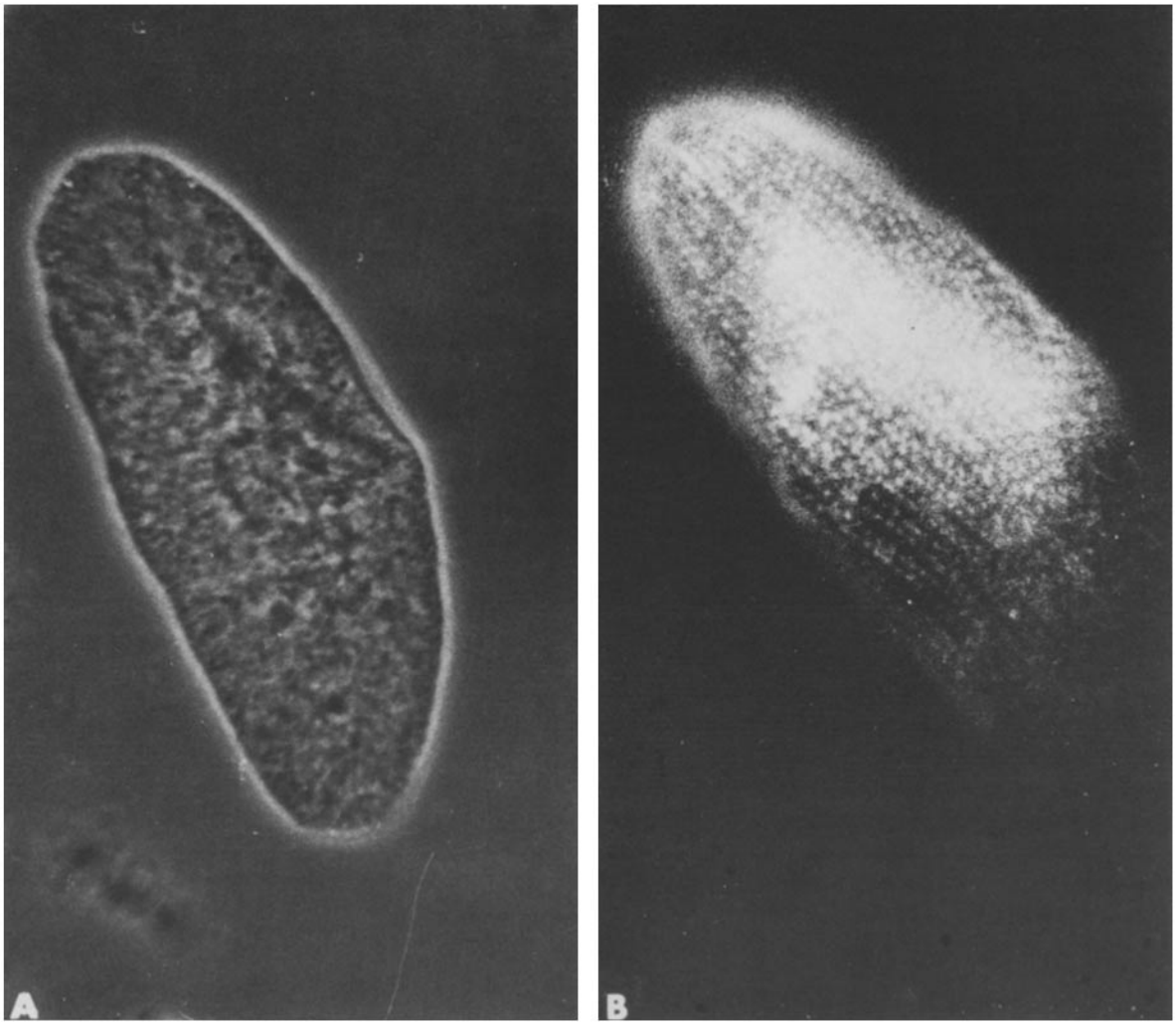


FIGURE 3 Fig. 3 *A* illustrates the phase contrast of the immunofluorescent image in Fig. 3 *B*. This deciliated cell clearly shows a linear punctuate array of fluorescent spots corresponding to calmodulin localization along the kineties of this cell. × 384.

light chain kinase (8, 41), and erythrocyte $Ca^{2+}$ ATPase (20). Calmodulin has also been implicated in regulation of a number of physiological events, including mitosis (39, 40), tubulin polymerization (25), endocytosis (31), and neurotransmitter release (12). Calmodulin is both ubiquitous and highly conserved, and has been described as the "2nd" second messenger (17); calmodulin is the most versatile physiologically of the calcium-binding proteins known to date.

Since the initial localization of calmodulin in the ciliated protozoan *Paramecium* (23), there have been a number of reports on the isolation and characterization of a calmodulin-like protein in *Tetrahymena* (27, 36, 29, 15, 18, 24). In the present study, we have identified a calmodulin-like protein in *Paramecium tetraurelia* using several different criteria. The ability of *Paramecium* extracts to stimulate mammalian PDE activity in a calcium-dependent manner, and the inhibition of this stimulation by trifluoperazine, is evidence for the presence of a polypeptide in this organism with properties similar to those of calmodulin. In addition, there exists an acidic, thermostable calcium-binding protein in *Paramecium* with an apparent molecular mass of 17,000 which migrates slightly faster than mammalian calmodulin standards in SDS PAGE, and whose mobility shifts in the presence of calcium. This relative shift in mobility in the presence of calcium has been suggested to be a reliable characteristic for detecting calmodulin in crude extracts using SDS PAGE analysis (3). Further, competitive binding of *Paramecium* heat-treated extract to a mammalian anticalmodulin describes a similar curve as rat testes heat-treated extract using the sensitive radioimmunoassay, thus indicating immunological cross-reactivity of anticalmodulin with a component in the *Paramecium* extract. Together, these data offer substantiative biochemical evidence for the presence of calmodulin in *Paramecium*.

Calmodulin appears to be localized within three discrete regions of *Paramecium*, resolvable at the light microscope level. First, indirect immunofluorescence results indicate that calmodulin is associated with large, spherical vacuolar inclusions. These cytoplasmic structures may represent food vacuoles of the cell, or perhaps calcium-containing birefringent vesicles present in certain ciliates.

A second important site of calmodulin localization in *Paramecium* is cilia. Calcium has been directly demonstrated to be involved in regulation of ciliary beat direction in *Paramecium* by Naitoh and Kaneko (28), working with Tritonated models. An increase in free calcium concentration from $10^{-7}$ to $10^{-6}$ M in ATP-containing reactivation solution causes first ciliary arrest and then a reversal in stroke direction. These responses form an important part of the swimming behavior of the living cell. Stimulation of the anterior portion of the swimming cell causes axonemal free calcium concentration to rise. The mech-

anism by which a rise in free calcium initiates the change in beat direction is unknown but could presumably involve calmodulin. Alternatively, calmodulin could be associated with calcium events in the ciliary membrane. Reed and Satir (30) have demonstrated reversal of the calcium arrest response in *Elliptio* cilia using trifluoperazine. Recently, Gitelman and Witman (14) demonstrated calmodulin stimulation of PDE by both flagellar membrane and axonemal preparations from *Chlamydomonas.* Furthermore, Blum and colleagues (1) have suggested that calmodulin confers calcium sensitivity to the ciliary ATPase, dynein.

Finally, a punctate pattern was observed in deciliated cells. It is important to keep the morphology of *Paramecium* in mind when discussing the punctate pattern of calmodulin localization. Within each cortical unit, several calcium-regulated events may occur including (*a*) changes in ciliary beat direction, (*b*) trichocyst release, and (*c*) endocytosis by way of parasomal sacs. Any or all of these calcium-mediated processes could involve calmodulin. It is probable that the fluorescent punctate pattern represents calmodulin localization in the remnant ciliary stubs or the underlying basal bodies which are contiguous with each cilium, since the correspondence of the fluorescent points with kineties is unmistakable. Both deciliated *Paramecium* and *Tetrahymena* exhibit this pattern of localization (23, 24). Typically, when cells are deciliated, the cilia detach between the region of the ciliary patches and the ciliary necklace and the basal bodies remain with the cell cortex (34). Interestingly, Anderson[2] in collaboration with Chafouleas and Means (unpublished data) has found that calmodulin comprises 1–2% of the total protein in isolated basal body preparations as determined by radioimmunoassay.

At this time we are unable to further resolve the specific localization of calmodulin within each cortical unit. However, we are currently pursuing the role of calmodulin in both *Paramecium* and *Tetrahymena* by determining which proteins in subcellular fractions of these cells will interact with calmodulin. We are also interested in the detailed structure of ciliate calmodulin. Information regarding the structural aspects of this protein in the ciliates, as well as its functional role in these organisms, may provide valuable insight into the evolution of calcium-binding proteins and into the role of calmodulin in calcium-mediated processes in eukaryotic cells.

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NOTE ADDED IN PROOF: Recently, Linden et al. (*Proc. Natl. Acad. Sci. U.S.A.* 78:308–312) have also demonstrated calmodulin is a component of isolated coated vesicles.

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