

Calmodulin Is a Major Component of Extruded Trichocysts from *Paramecium tetraurelia*

JAMES J. RAUH and DAVID L. NELSON

Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

ABSTRACT Extruded trichocysts are composed of a family of proteins with molecular weights between 15,000 and 20,000. We have used heat treatment and affinity chromatography on fluphenazine-Sepharose to purify calmodulinlike proteins from whole cells and from extruded trichocysts. The purified protein from trichocysts is indistinguishable from that of whole cells; it is heat-stable, activates brain phosphodiesterase in a Ca^{++} -dependent fashion, changes mobility on SDS polyacrylamide gels in the presence of Ca^{++} , contains 1 mol of trimethyllysine/17 kdaltons, and has the amino acid composition characteristic of calmodulins. Calmodulin is a major component of purified, extruded trichocysts, of which it represents between 1 and 10% by mass. The other proteins of the trichocyst also resemble calmodulin in several properties. Possible roles for calmodulin in the Ca^{++} -activated extrusion of trichocysts is discussed.

Trichocysts are specialized secretory organelles found in *Paramecium*, each enclosed in membranous vesicles lying just beneath the cell surface (1, 2). In response to various stimuli, these vesicles fuse with the cell surface and their contents decondense explosively, producing needlelike extracellular structures up to 40 μm long. The function of trichocysts is uncertain, but their morphology (3-5) and the genetics of their development (2, 4, 6-8) and extrusion (9-14) have been extensively studied. The decondensation process apparently requires Ca^{++} (9, 15). Steers et al. (3) showed that the extruded trichocysts were composed of a protein, "trichynin," of ~17 kdaltons. We (16) resolved extruded trichocysts into a family of acidic peptides of 15-20 kdaltons. We show here that one of these major components of extruded trichocysts is calmodulin, a widely distributed Ca^{++} -binding protein known to mediate the regulation of a wide variety of enzymes by Ca^{++} (17, 18).

MATERIALS AND METHODS

Paramecium tetraurelia wild-type stock 51S (nonkappa bearing) was grown axenically at 28°C in the Soldo crude medium (19) modified as described by Thiele et al. (20). Cells were grown to late logarithmic phase (30,000 cells/ml). The "trichless" (lacking trichocysts) mutant strain (t1) used (provided by C. Kung, University of Wisconsin-Madison) as grown at 28°C in phosphate-buffered Cerophyl medium, modified as described by Hansma (21), and bacterized with *Enterobacter aerogenes* (22).

Cells were harvested by centrifugation at 170 g_{max} , washed in room-temperature Dryl's solution (1 mM NaH_2PO_4 , 1 mM Na_2HPO_4 , 2 mM Na_3 citrate, 1.5 mM CaCl_2 , pH 6.9) (23) to remove all traces of medium, and pelleted at 200 g_{max} for 2 min. using pear-shaped oil-testing centrifuge tubes. To purify trichocysts from these cells, we washed them in 4°C Dryl's solution which induced trichocyst firing. A 2-min centrifugation at 200 g_{max} pelleted the cells and left the trichocysts as a distinct fluffy layer on top of the cells. The trichocysts were removed and the cells were washed until no further trichocyst release occurred. The resulting

trichocysts were centrifuged several times at 850 g_{max} for 2 min to sediment any contaminating whole cells. The trichocysts, which were present in the resulting supernate, were recovered by centrifugation at 27,000 g_{max} for 20 min. The trichocysts were resuspended in 10 mM Tris-Cl, pH 8.0, and 0.2- to 0.3-ml aliquots were loaded onto the sucrose step gradient, described previously (16). The gradient was centrifuged at 243,000 g_{max} for 1.5 h at 4°C. Trichocysts, which banded at the interface of the 55 and 66% sucrose layers, were removed and washed by centrifugation in 25 vol of 10 mM Tris-Cl, pH 8.0.

Purification of Calmodulin from Whole Cells and Trichocysts

Whole cells in 4°C Dryl's solution were disrupted with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, N. Y.). Cell debris was removed by centrifugation at 27,000 g_{max} for 30 min. The supernate was placed in a boiling water bath for 10 min. The maximum temperature reached by the supernate was 85°C. Precipitated protein was removed by centrifugation at 48,000 g_{max} for 30 min. The supernate was dialyzed against 10 mM Tris-Cl, 0.5 mM CaCl_2 , pH 7.0, for 48 h and then concentrated by using an Amicon ultrafiltration cell with a PM-10 membrane (Amicon Corp., Lexington, Mass.). Trichocysts were extracted by the same procedure as whole cells, except that the homogenization and ultrafiltration steps were omitted. The fluphenazine-Sepharose affinity resin was prepared by the method described by Sundberg and Porath (24) and later used successfully by Charbonneau and Cormier (25). Fluphenazine was coupled to the Sepharose 4B by means of the 12-atom spacer, 1,4-butanediol diglycidyl ether.

The heat-stable proteins from whole cells or trichocysts were loaded onto a 9-ml column (1 cm x 11 cm) containing fluphenazine-Sepharose 4B equilibrated with 10 mM Tris-Cl, 0.5 mM CaCl_2 , pH 7.0 (column equilibration buffer), at 4°C, and the column was washed with 2 bed vol of the column equilibration buffer, followed by 2 bed vol of the column equilibration buffer containing 0.5 M NaCl, and a final wash with 2 bed vol of 10 mM Tris-Cl, 5 mM EGTA, pH 8.0. 1-ml fractions were collected at a rate of 15 ml/h. Protein concentrations were determined by the method of Lowry et al. (26).

Samples were heated at 100°C for 2 min and loaded onto the gel. For "calcium-shift" gels the sample contained either 5 mM CaCl_2 or 5 mM EGTA. Gels were run at 20 mA and stopped just as the dye-front left the bottom of the

gel. Molecular weight standards (all from Sigma Chemical Co., St. Louis, Mo.) were as follows: conalbumin (86,000), bovine serum albumin (68,000), ovalbumin (43,000), α -chymotrypsinogen A (26,000), myoglobin (17,000), and dansylated cytochrome *c* (15,000).

The assay for phosphodiesterase stimulation was modified from Teo et al. (27). Calmodulin was added to a reaction mixture containing 25 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM cAMP, 50 μ g (0.007 U) cyclic nucleotide phosphodiesterase in a final volume of 1 ml. This was incubated at 30°C for 15 min and quenched by heating at 100°C for 2 min. To release inorganic phosphate (P_i) from 5'-nucleotides, snake venom (*Crotalus atrox*) (50 μ g) was added to the reaction mixture and incubated at 37°C for 30 min. This reaction was quenched with the addition of 0.2 ml of 32.5% TCA. P_i was measured by the method of Chen et al. (28).

Fluphenazine-2HCl was provided by E. R. Squibb and Sons, Inc. (Princeton, N. J.). Bovine testis calmodulin was a gift from F. Siegel, University of Wisconsin-Madison. 3',5'-cyclic nucleotide phosphodiesterase (calmodulin sensitive) was obtained from Sigma Chemical Co.

RESULTS

Purification of Calmodulin from Whole Cells by Affinity Chromatography

When a homogenate of whole cells was heated for 10 min at 85°C and centrifuged at 48,000 g_{max} for 30 min, ~20% of the cell protein remained in the supernate (Table I). Most of the protein remaining after dialysis of this heat-stable fraction passed directly through the fluphenazine-Sepharose affinity column and was eluted as a broad peak (peak I, Fig. 1a). Nearly all the protein which adsorbed to the column could be eluted by a buffer containing 0.5 M NaCl (peak II, Fig. 1a). This second peak generally represented 5–20% of the total protein eluted, and SDS PAGE revealed a complex but reproducible pattern of polypeptides. The binding of calmodulin to fluphenazine is Ca⁺⁺-dependent and to some extent pH-dependent (29). Elution with a pH 8.0 buffer containing EGTA released a small amount of protein (~1% of the total eluted) from the affinity column (peak III, Fig. 1a), which consisted of a single polypeptide with the properties of calmodulin (see below).

Purification of Calmodulin from Extruded Trichocysts

Extruded trichocysts from the sucrose gradient (Materials

TABLE I
Protein Recoveries during Calmodulin Purification

	Whole cells		Trichocysts	
	Protein* mg	% of total‡	Protein§ mg	% of total‡
Initial material	1,700	100	56	100
Heat-treated supernate	300	18	9	16
Post-dialysis concentrate	36	2	2.6	5
Column fractions:				
I	19	1.1 (84)	0.1	0.2 (6)
II	3.4	0.2 (15)	1.2	2.1 (67)
III	0.3	0.02 (1)	0.5	0.9 (28)

This table represents data from a typical experiment. This procedure has been repeated 16 times with closely similar results. The preparation of whole cells and trichocysts shown above was chosen because it corresponds to the elution profiles shown in Fig. 1.

* This amount of protein was obtained from 1.6×10^9 cells.

‡ The numbers in parentheses represent the percentage of total protein eluted from the affinity column.

§ This amount of protein was obtained from 4.0×10^9 cells.

and Methods) were essentially free of contaminating material as judged by phase-contrast microscopy and electron microscopy of negatively stained preparations (Fig. 2a). Most of the trichocysts had separated from their tips, but the preparation contained roughly equivalent numbers of tips and shafts. Most of the visible structures had the characteristic 55-nm periodicity and were ~230 nm wide (Fig. 2b), but in some instances the trichocyst shafts seemed to have flattened or unrolled and to have splayed-out ends (Fig. 2c). SDS PAGE gave the polypeptide pattern we reported previously (16): two major bands near 17 kdaltons, and several less prominent bands between 15 and 20 kdaltons (Fig. 2d).

Treatment at 85°C solubilized the purified trichocysts as expected from the work of Pollack and Steers (30) (Table I). The profile of trichocyst protein eluted from the fluphenazine affinity column (Fig. 1b) was strikingly different from that of "whole-cell" protein: very little of the applied protein passed directly through the column (peak I, Fig. 1b). Washing the column with high salt did remove a significant amount of protein (peak II, Fig. 1b), and a relatively large peak, representing ~28% of the total protein recovered, was eluted with EGTA (peak III, Fig. 1b).

SDS PAGE Analysis of Trichocyst Fractions

Washed, extruded trichocysts showed the polypeptide pattern described above on polyacrylamide gels in SDS: a set of low-molecular-weight bands centered around 17 kdaltons (lane b, Fig. 3). Most trichocyst preparations also contained a less prominent band of about 68 kdaltons (lane b, Fig. 3). The peptide pattern of protein solubilized by heat treatment (lane c, Fig. 3) was similar to, but not identical with, that of whole trichocysts (lanes b and c, Fig. 3). Dialysis of the heat-stable supernate did not appreciably change the peptide pattern (lane

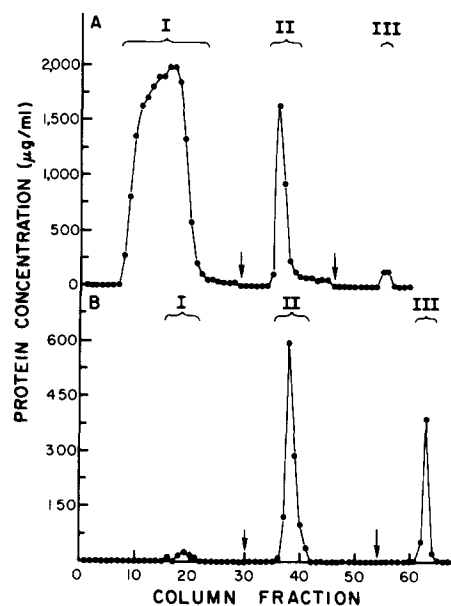


FIGURE 1 Affinity chromatography of a *Paramecium* and trichocyst extract (heat stable) on a fluphenazine-Sepharose 4B column. (A) 36 mg of whole-cell protein and (B) 2.6 mg of trichocyst protein were loaded onto columns beginning with fraction 1. The columns were washed with 10 mM Tris-Cl, 0.5 mM CaCl₂, 500 mM NaCl, pH 7.0, beginning with fraction 30 (arrows). At about fraction 50, the columns were washed with 10 mM Tris-Cl, 5 mM EGTA, pH 8.0 (arrows). The fractions containing protein were pooled as indicated in the figure.

d, Fig. 3), although a substantial fraction of the protein was lost during dialysis (Table I), probably by precipitation. Affinity chromatography resolved the dialyzed, heat-stable extract into fractions with distinctly different polypeptide compositions (lanes e-g, Fig. 3). Peak I (lane g) contained relatively little of the major 17-kdalton species and was enriched for a slightly slower-migrating peptide; peak II (lane f) contained all of the peptides of the dialyzed fractions and in similar proportions; and peak III (lane e) was almost exclusively a polypeptide of 17 kdaltons, one of the two peptides in the prominent doublet of whole trichocysts.

One diagnostic feature of calmodulin is its increased mobility in the presence of Ca^{++} on SDS polyacrylamide gels. The putative calmodulin fraction purified from whole cells of *Paramecium* did show this Ca^{++} -dependent mobility change (lanes a and b, Fig. 4) as did bovine calmodulin (lanes e and f, Fig. 4). The peak III protein from trichocysts showed a clear increase in mobility in the presence of Ca^{++} (lanes c and d, Fig. 4). The peptides of whole trichocysts showed little if any change in mobility with Ca^{++} (lanes g and h, Fig. 4).

Ca^{++} -dependent Activation of Brain Phosphodiesterase

Calmodulins from a variety of sources activate the cyclic nucleotide phosphodiesterase (PDE) of brain in a Ca^{++} -dependent manner (17). The peak III protein from whole cells and that from trichocysts also stimulated brain PDE (Table II). Both EGTA and the calmodulin antagonist trifluoperazine blocked this stimulation. The amount of trichocyst calmodulin needed for maximal stimulation was the same as the amount of bovine calmodulin. Trichocyst calmodulin itself showed no PDE activity.

Amino Acid Composition of Calmodulins from *Paramecium*

The amino acid composition of calmodulin purified from whole paramecia is indistinguishable from that of trichocyst calmodulin, and is closely similar to that of calmodulin from *Tetrahymena* and bovine testis (Table III). The *Paramecium* proteins, like calmodulins from other sources, are rich in acidic

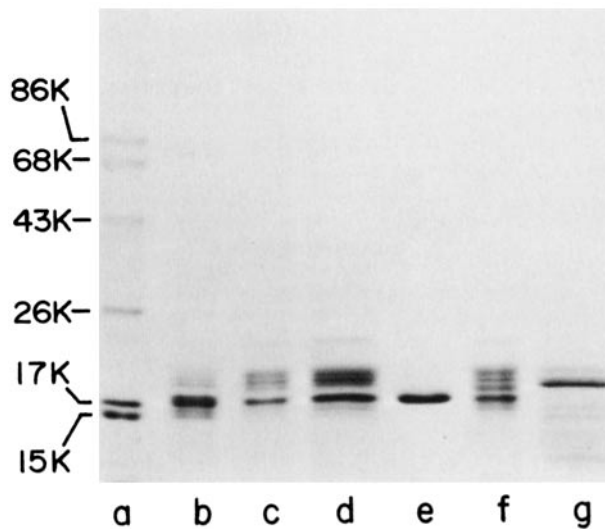


FIGURE 3 SDS polyacrylamide gel of trichocyst proteins during calmodulin purification. (a) Molecular weight standards; (b) whole trichocysts, 50 μg ; (c) heat-stable trichocyst proteins, 50 μg ; (d) heat-stable proteins after dialysis and concentration, 40 μg ; (e) peak III of affinity column, 10 μg ; (f) peak II, 25 μg ; and (g) peak I, 25 μg .

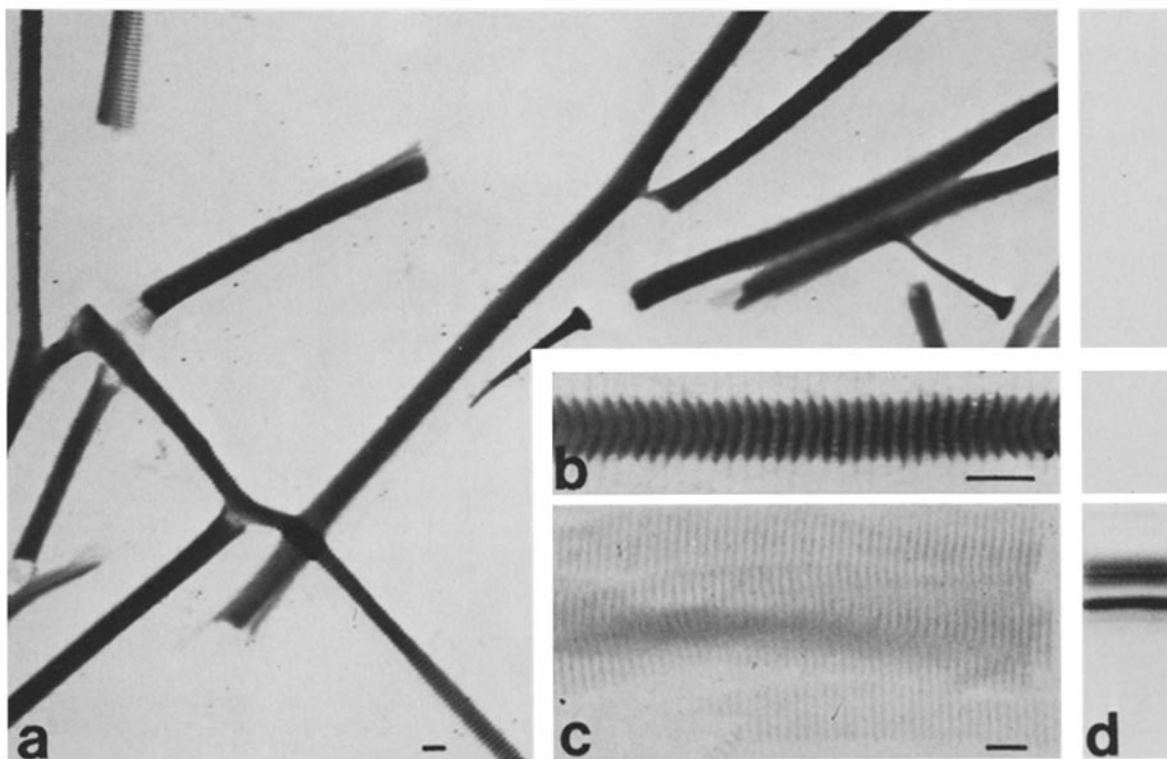


FIGURE 2 Transmission electron micrographs of purified trichocysts stained in 1% phosphotungstic acid for 3 min. (a) Extruded trichocyst shafts and tips, (b) a portion of the trichocyst shaft at high magnification, (c) a section of the trichocyst shaft that has unrolled, and (d) peptide pattern exhibited by this trichocyst preparation on SDS polyacrylamide gels. Bars, 0.25 μm .

amino acids, lack cysteine, have a high phenylalanine to tyrosine ratio, and contain a single residue of trimethyllysine per 17 kdaltons.

What Fraction of Trichocyst Protein Is Calmodulin?

The recovery of calmodulin during purification (Table I) provides a minimum estimate of the amount of calmodulin in trichocysts; ~1% of the total protein of trichocysts is recovered as calmodulin. The purification steps before affinity chromatography do not change the SDS PAGE pattern very significantly (Fig. 3), but recovery of trichocyst protein in the dialyzed heat-stable fraction is only 5%. If this loss of protein is nonspecific, the actual amount of calmodulin could be 20 times more than we recover from the affinity column, or ~20%. To resolve this question, we analyzed the amino acid compo-

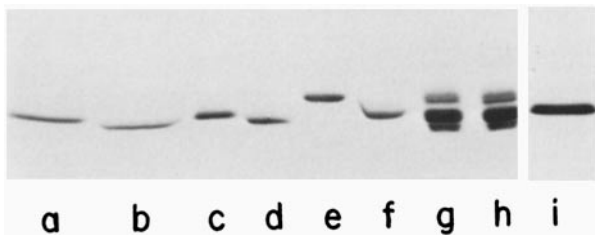


FIGURE 4 Ca^{2+} -dependent mobility shifts of calmodulin on SDS polyacrylamide gels. Samples in lanes a, c, e, g, and i contain 5 mM EGTA. Samples in lanes b, d, f, and h contain 5 mM CaCl_2 . (a and b) Whole-cell calmodulin, 10 μg ; (c and d) trichocyst calmodulin, 10 μg ; (e and f) bovine calmodulin, 10 μg ; (g and h) whole trichocysts, 50 μg ; (i) calmodulin from trichless mutant, 10 μg .

TABLE II
Phosphodiesterase Stimulation by *Paramecium* Calmodulin

Source	Fold stimulation
Whole cell calmodulin	
5 μg (+EGTA)	1.3
2 μg	1.8
5 μg	2.0
10 μg	2.0
Whole cell calmodulin (5 μg + trifluoperazine)	
0 μM TFP	2.1
1 μM TFP	1.6
2 μM TFP	1.3
5 μM TFP	1.0
Trichocyst calmodulin	
5 μg (+EGTA)	0.9
2 μg	1.9
5 μg	2.2
10 μg	2.1
Bovine calmodulin	
5 μg (+EGTA)	1.5
2 μg	1.1
5 μg	2.1
10 μg	2.3

These assays were performed as described in Materials and Methods using 50 μg of bovine heart PDE. Under these assay conditions, PDE activity was linear with respect to time and protein. In this experiment, maximal stimulation corresponded to a specific activity of 35 nmol $\text{P}_i/\text{min} \times \text{mg}$. This basic experiment has been repeated four times. While each experiment gives results which are internally consistent with the results shown above, the separate experiments can not readily be compared statistically due to a decrease in the fold-stimulation of the PDE upon storage at -20°C . In fresh preparations of PDE, stimulation of up to fivefold by calmodulin was observed.

TABLE III
Amino Acid Composition of *Paramecium* Calmodulin

	Whole cell calmodulin* \ddagger	Trichocyst calmodulin*	Tetrahy- mena calmodulin \S	Bovine calmodulin \parallel	Whole trichocysts*
Aspartic acid	21.1	21.9	23	23	20.3
Threonine	9.3	9.4	11	12	8.6
Serine	5.4	4.9	4	4	8.2
Glutamic acid	29.0	28.3	25	27	32.8
Proline	2.5	2.3	2	2	1.7
Glycine	12.0	11.5	11	11	4.6
Alanine	11.5	11.4	11	11	18.0
Valine	6.8	6.7	6	7	7.6
Half-cystine	0.0	0.0	0	0	1.4
Methionine	7.4	7.6	8	9	0.7
Isoleucine	8.3	8.5	9	8	6.7
Leucine	12.5	12.4	12	9	15.1
Tyrosine	1.4	1.1	1	2	3.3
Phenylalanine	8.1	8.0	8	8	3.9
Lysine	6.9	6.3	7	7	7.3
Histidine	2.7	2.8	2	1	2.6
Arginine	5.7	6.0	6	6	8.9
Trimethyllysine	1.0	1.1	1	1	≤ 0.1
Total Residues	151.6	150.2	147	148	151.7

Amino acid compositions were determined after a 24-h hydrolysis at 110°C in 6N HCl, 0.2% Phenol. Samples were run on a Dionex D500 Automatic Amino Acid Analyzer.

* Residues/molecule computed on the basis of 17,000 g/mol.

\ddagger This column represents the mean of four different whole cell calmodulin preparations.

\S From reference 31.

\parallel From reference 32.

sition of whole trichocysts. Trimethyllysine, the amino acid present at one residue per molecule in most calmodulins, represented <0.1 residue per 17 kdaltons of whole trichocyst protein (Table III). If all the protein of trichocysts is ~17 kdaltons, and if only calmodulin contains trimethyllysine, then calmodulin represents between 1 and 10% of total trichocyst protein.

Another estimate of the maximum calmodulin content of trichocysts is based on the relatively high methionine content of purified calmodulin (8 residues/17 kdaltons) which is 10 times higher than that of whole trichocysts. Calmodulin could, therefore, make up as much as 10% of the total protein of isolated trichocysts, but not more.

Calmodulin Content of Cells with and without Trichocysts

Washing paramacia in cold buffer results in extrusion of virtually all mature trichocysts, but a similar wash with warm buffer causes very little trichocyst firing. We purified calmodulin from cells washed each way and found that cells washed in the cold (and therefore lacking trichocysts) yielded 40–50% as much calmodulin (peak III) as cells washed at room temperature. Immature trichocysts are presumably not fired in this cold wash procedure and could therefore account for much of the calmodulin remaining in cold-washed cells.

We examined a mutant (t1) in which trichocyst development is interrupted at an early stage and in which no trichocysts are detectable by electron microscopy (4). From this mutant we recovered approximately the same amount of calmodulin in

peak III of the affinity column (lane *i*, Fig. 4) from whole cell extracts as was recovered from wild-type cells.

DISCUSSION

The protein we have purified from whole cells and extruded trichocysts of *Paramecium* by fluphenazine affinity chromatography has the properties characteristic of calmodulin from many other sources. It is a heat-stable, acidic protein of ~17 kdaltons which contains 1 mol of trimethyllysine per mole of protein; its mobility on SDS gels increases in the presence of Ca^{++} ; it activates brain PDE in a Ca^{++} -dependent manner, and this stimulation is blocked by trifluoperazine for which the protein has Ca^{++} -dependent affinity. We therefore consider it appropriate to call this protein calmodulin.

Paramecium calmodulin appears to be a major component of extruded trichocysts since it represents 1–10% by mass, a value which is 50- to 500-fold higher than the yields achieved from the whole cells of *Paramecium* and other protozoans. The observation that cells washed free of mature trichocysts contain only 50% as much calmodulin as cells retaining their trichocysts indicates that a large proportion of the calmodulin of the whole cell is part of this organelle.

The other proteins of trichocysts may be related to calmodulin; they bind loosely to the fluphenazine affinity column, are of ~17 kdaltons in weight, and are acidic as judged by isoelectric focusing (16) and by the high aspartic and glutamic acid content of the whole trichocyst.

The trichless mutant was found to have a normal calmodulin content. This is not surprising since the defect in this mutant is in the assembly of the trichocyst organelle. Most, if not all, of the trichocyst components could be present at normal levels without being assembled properly.

The firing of trichocysts, which occurs within milliseconds, involves membrane fusion and exocytosis, as well as a reorganization which leads to a sevenfold increase in length of the shaft of the trichocyst. The former generally involves Ca^{++} , and the latter, which can be observed *in vitro*, is also apparently Ca^{++} -dependent (9, 15). Satir et al. (33) have noted that the calmodulin antagonist trifluoperazine blocked trichocyst extrusion. In similar experiments, we have been unable to observe any effects of phenothiazines on trichocyst extrusion at drug concentrations which are compatible with cell survival. Plattner and co-workers (12, 15) have shown that agents known to interfere with Ca^{++} fluxes across membranes affect trichocyst firing.

Stimuli which lead to trichocyst firing may do so by triggering Ca^{++} influx into the region where mature trichocysts rest against the plasma membrane. The local increase in Ca^{++} might be detected by calmodulin, which could then activate some other protein essential in trichocyst firing, in a manner analogous with its role in modulating other Ca^{++} -dependent enzymes.

The large amount of calmodulin in trichocysts suggests an alternative role for it: it may be a structural rather than a catalytic component of the trichocyst. The enormous expansion of the trichocyst triggered by Ca^{++} might then be the result of a conformational change in calmodulin upon Ca^{++} binding, which results in polymerization of monomers or "stretching" of preexisting aggregates. Calmodulin is known to influence the state of aggregation of tubulin in microtubules in a Ca^{++} -dependent manner (18).

We have recently found that the release of mucocysts (organelles similar to trichocysts) from *Tetrahymena* yields a

family of low-molecular-weight polypeptides similar to those of trichocysts (manuscript in preparation), suggesting that calmodulinlike proteins may play a general role in exocytotic events in protozoa.

Calmodulin very likely plays other roles in *Paramecium*. We have discovered that *Paramecium* contains a second calmodulinlike protein with properties similar to those of the calmodulin described here, and its relationship to trichocyst calmodulin is now being explored. We have reported that calmodulin antagonists block the Ca^{++} -dependent ciliary reversal mechanism in *Paramecium* (34), and it is possible that a calmodulinlike protein is involved in that pathway.

Walter and Schultz (35) have recently described a calmodulinlike protein purified from whole cells of *P. tetraurelia* by procedures not involving affinity chromatography. Although the amino acid composition of their calmodulin was not determined, its other properties are very similar to those we have described here for whole cell and trichocyst calmodulin. Part of the calmodulin they purified from whole cells no doubt originate from trichocysts. Walter and Schultz (35) also found calmodulin in the cilia, confirming the immunohistochemical results of Maihle et al. (36), who also showed that extracts of whole paramecia contained calmodulin which showed the same properties we describe here. Blum et al. (37) have recently found a calmodulin associated with ciliary dynein and have suggested that it may play a role in the Ca^{++} regulation of ciliary beating.

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