Calmodulin Confers Calcium Sensitivity on Ciliary Dynein ATPase

J. J. BLUM, ALVERNON HAYES, GORDON A. JAMIESON, Jr., and THOMAS C. VANAMAN
Departments of Physiology and of Microbiology and Immunology, Duke University, Durham, North Carolina 27710

ABSTRACT Extraction of demembranated cilia of *Tetrahymena* by Tris-EDTA (denoted by the suffix E) yields 14S-E and 30S-E dyneins with ATPase activities that are slightly increased by Ca^{++}. This effect is moderately potentiated when bovine brain calmodulin is added to the assay mixture. Extraction with 0.5 M KCl (denoted by the suffix K) yields a 14S-K dynein with a low basal ATPase activity in the presence of Ca^{++}. Subsequent addition of calmodulin causes marked activation (up to 10-fold) of ATPase activity. Although 14S-K and 14S-E dyneins have Ca^{++}-dependent ATPase activities that differ markedly in the degree of activation, the concentration of calmodulin required for half-maximal saturation is similar for both, ~0.1 μM. Both 30S-K and 30S-E dyneins, however, require ~0.7 μM bovine brain calmodulin to reach half-maximal activation of their Ca^{++}-dependent ATPase activities. *Tetrahymena* calmodulin is as effective as bovine brain calmodulin in activating 30S dynein, but may be slightly less effective than the brain calmodulin in activating 14S dynein. Rabbit skeletal muscle troponin C also activates the Ca^{++}-dependent ATPase activity of 30S dynein and, to a lesser extent, that of 14S dynein, but in both cases is less effective than calmodulin.

The interaction of calmodulin with dynein that results in ATPase activation is largely complete in <1 min, and is prevented by the presence of low concentrations of ATP. Adenylyl imidodiphosphate can partially prevent activation of dynein ATPase by calmodulin plus Ca^{++}, but at much higher concentrations than required for prevention by ATP. β,γ-methyl-adenosine triphosphate appears not to prevent this activation.

The presence of Ca^{++}-dependent calmodulin-binding sites on 14S and 30S dyneins was demonstrated by the Ca^{++}-dependent retention of the dyneins on a calmodulin-Sepharose 4B column. Gel electrophoresis of 14S dynein that had been purified by the affinity-chromatography procedure showed the presence of two major and one minor high molecular weight components. Similar analysis of 30S dynein purified by this procedure also revealed one major and one minor high molecular weight components that were different from the major components of 14S dynein.

Ca^{++}-dependent binding sites for calmodulin were shown to be present on axonemes that had been extracted twice with Tris-EDTA or with 0.5 M KCl by the use of ^{35}S-labeled *Tetrahymena* calmodulin. It is concluded that the 14S and 30S dyneins of *Tetrahymena* contain Ca^{++}-dependent binding sites for calmodulin and that calmodulin mediates the Ca^{++}-regulation of the dynein ATPases of *Tetrahymena* cilia.

A key role for Ca^{++} in the control of ciliary and flagellar motility has been well documented (see reference 13 for a recent review). Most of the effects of Ca^{++} have been studied in intact systems, where Ca^{++} influx and efflux through the membrane was demonstrated to control the direction and/or frequency of beating. Several effects of Ca^{++} on demembranated axonemal preparations have also been observed. These include reversal of direction of wave propagation in flagella of *Crithidia* (26), reversal of direction of beat in cilia of *Paramecium* (37), and an increase in the pellet height response of

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demembranated cilia of *Tetrahymena* (8). However, the molecular mechanisms responsible for the effect of Ca^{++} on the axonemal components are unknown. The recent finding that *Tetrahymena* contain calmodulin (28, 31) and that part of this calmodulin is in ciliary axonemes and is associated with the 14S dynein fraction (28), suggested that calmodulin might confer Ca^{++}-sensitivity on the 14S dynein—possibly on its ATPase activity. Preliminary studies showed that the ATPase activity of crude dynein obtained by Tris-EDTA extraction was indeed enhanced by addition of Ca^{++} if additional calmodulin was present, but the effects were small. Because Doughty (17) has used a KCl extraction procedure to observe the effects of low concentrations of Ca^{++} on Mg^{++}-dependent dynein ATPase activity, it seemed possible that a KCl extraction procedure might be more effective in stripping calmodulin from the dyneins and, hence, in yielding dyneins that were sensitive to Ca^{++} only in the presence of added calmodulin. Greater sensitivity to calmodulin was indeed observed after KCl extraction and it is that sensitivity to Ca^{++} in the presence of added calmodulin which initiated the studies presented in this report.

MATERIALS AND METHODS

Materials

2-Chloro-10-(3-aminopropyl)phenothiazine (CAPP),[1] a generous gift from Dr. Carl Kaiser of the Smith, Kline and French Laboratories, Philadelphia, Pa., was coupled to Sepharose 4B and the resulting CAPP-Sepharose 4B conjugate prepared for use as previously described (27) and used as detailed below under the headings Calmodulin Purification and Affinity Chromatography. Calmodulin-Sepharose 4B was prepared, characterized, and used as previously described (48). Sephadex gel filtration media were prepared for use as described by Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. SDS, acrylamide, and bisacrylamide were used as supplied by BDH Chemicals, Ltd., Poole, England; *N,N,N',N'*-tetramethylenediamine (TEMED) and 2-mercaptoethanol as supplied by Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.; and Tris, imidazole, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), chlorpromazine (CPZ), 2-mercaptoethanol, phenylmethylsulfonylfluoride (PMSF), AMP-PNP, and Coomassie Brilliant Blue R as supplied by Sigma Chemical Co., St. Louis, Mo. AMP-PCP was used as supplied by P-L Biochemicals, Inc., Milwaukee, Wisc., and EDTA as supplied by J. T. Baker Chemical Co., Phillipsburg, N. J. Leupeptin was obtained from the Peptide Institute, Inc., Osaka, Japan. Urea solutions (Fisher Scientific Co., Pittsburgh, Pa.) were deionized immediately before use. Reagents for amino acid analysis (45) and ATPase assays (9) were as previously described. ATP was obtained from Pabst Research Laboratories, Milwaukee, Wisc. Rabbit skeletal muscle troponin C (TnC) was prepared according to Perry and Cole (39). Protein M_r standards were from Bio-Rad Laboratories, Richmond, Calif., and contained High Molecular Weight Standards: myosin (200,000), β-galactosidase (116,500), phosphorylase *b* (94,000), bovine serum albumin (68,000), and ovalbumin (43,000), or Low Molecular Weight Standards: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300).

Analytical Procedures

Procedures for measurement of protein concentration were as described (10), except that the concentrations of bovine brain and *Tetrahymena* calmodulin and of TnC stock solutions were determined by amino acid analysis after acid hydrolysis (45). ATPase activity was assayed at pH 7.5 for 20 min at 25°C in a total volume of 1.0 ml and a final concentration of 1.0 mM ATP unless otherwise specified, as described earlier (9). All assays were done in duplicate, and reproducibility was within ±5%. At the dynein concentrations used, phosphate release was linear over the course of the assay. Gel electrophoretic procedures were performed as detailed in the text and figure legends.

Calmodulin Purification

^{35}S-LABELED TETRAHYMENA CALMODULIN: Two cultures of 100 ml each of *Tetrahymena* were grown to early stationary phase ($\sim$528,000 cells/ml). The cells were collected by centrifugation for 3 min at 900 *g* at room temperature and resuspended at a density of $\sim$140,000 cells/ml in 60 ml of Wagner's salt solution (43) at 25°C. A culture of *E. coli* Na-22 (14) containing $\sim$5 mCi of incorporated [^{35}S]sulfate (the generous gift of Dr. F. H. Schachat, Duke University, Durham, N. C.) was then added to the Erlenmeyer flask containing the *Tetrahymena*, and the mixed cultures were incubated at 25°C with shaking. No bacteria were observed after 20 min. Shaking was continued for an additional 3 h, at which time the cells were pelleted and frozen until processed as described below.

Purification of ^{35}S-labeled *Tetrahymena* calmodulin was generally as previously described (27). Cells were homogenized in two volumes of 20 mM TES-NaOH/1 mM 2-mercaptoethanol/1 mM EDTA (pH 7.0) containing 1 mM PMSF and 2.5 mg/liter leupeptin (4). The homogenate was centrifuged at 10,000 *g* for 30 min and the resulting supernate processed batchwise onto 10 g of DEAE-cellulose (DE-52, Whatman Inc., Clifton, N. J.) that had been equilibrated with homogenization buffer. The resin was washed twice with 100 ml of homogenization buffer containing 0.1 M NaCl and the resin-bound material was then eluted with 100 ml of homogenization buffer containing 0.5 M NaCl. After addition of CaCl$_2$ (to 5 mM) to the high-salt eluant, it was applied to a CAPP-Sepharose 4B column (27) of 25-ml bed volume. The affinity column was then washed with 100 ml of homogenization buffer containing 0.5 M NaCl and 5 mM CaCl$_2$. Material bound in a Ca^{++}-dependent manner to this affinity column was eluted with homogenization buffer containing 0.5 M NaCl and 10 mM EGTA. Fractions containing EGTA-eluted radioactive material were pooled, Ca^{++} in excess of the EGTA present in the elution buffer added, and the pooled material dialyzed against 1 mM NH$_4$HCO$_3$/1 mM 2-mercaptoethanol and finally freeze-dried. The freeze-dried material was dissolved in 0.5 ml of 10 mM NH$_4$HCO$_3$ and subjected to gel filtration on a column (1.5 × 130 cm) containing Sephadex G-100, using 10 mM NH$_4$HCO$_3$ as the eluant. Fractions (2 ml) were analyzed for radioactivity and by electrophoresis on alkaline urea/10% polyacrylamide gels (23), [^{35}S]calmodulin being detected by Coomassie Blue staining and by radiography of the gel after drying. Fractions containing homogeneous [^{35}S]calmodulin were pooled and freeze-dried, and a portion was used to study the binding of calmodulin to twice-extracted axonemes, as described below. The specific activity of the [^{35}S]*Tetrahymena* calmodulin ($\sim 1.3 \times 10^5$ cpm/μg) was obtained by subjecting a sample containing a known number of counts to alkaline urea-PAGE (polyacrylamide gel electrophoresis) and estimating the protein concentration of the calmodulin band by the level of Coomassie Blue staining (45).

UNLABELED CALMODULINS: *Tetrahymena* calmodulin was isolated exactly as described above for the isolation of radiolabeled material, except on a larger scale. Bovine brain calmodulin was also purified with this procedure, except that ion-exchange chromatography on DEAE-Sephadex A-50, as described previously (27, 45) was inserted between the CAPP-Sepharose 4B affinity chromatography and Sephadex G-100 gel filtration steps. The yields of *Tetrahymena* and of bovine brain calmodulin obtained by these procedures were significantly greater than those previously described (28, 45, 46). These methods represent those currently in use in our laboratories for the purification of calmodulin.

Cilia Preparation and Extraction

Cilia were prepared from cultures of *Tetrahymena pyriformis* strain HSM and demembranated with 0.05% Triton X-100 as described previously (7). The procedures for preparation of crude dynein by extraction of the axonemes with Tris-EDTA (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.2) and for preparation of 30S and 14S dyneins from the crude dynein by sucrose density gradient sedimentation have also been described (6, 7). KCl extraction procedures were performed as follows: The demembranated axonemes were resuspended in IMT/6 buffer (8.33 mM Tris-HCl/8.33 mM imidazole/1.25 mM MgCl$_2$/0.067 mM EGTA, pH 7.5) containing 0.15 M, 0.3 M, or 0.5 M KCl and gently stirred at 4°C for the times specified in the text. The suspension was then centrifuged (12,000 *g* for 10 min for the 0.15 or 0.30 M KCl extractions, 27,000 *g* for 20 min for the 0.5 M KCl extraction) yielding a supernatant fraction, Si, and a pellet fraction, Pi, where i is the number of such extraction steps. The supernates from the 0.5 M KCl extraction step were resolved by sucrose density gradient centrifugation exactly as for crude dynein obtained by the Tris-EDTA extraction procedure. To distinguish between fractions obtained by Tris-EDTA extraction and fractions obtained by KCl extraction, we shall use the suffixes E and K, respectively. Thus, P1-E is the pellet fraction and S1-E the supernatant fraction obtained after one extraction step with Tris-EDTA, whereas P1-K and S1-K are obtained after one extraction with a specified concentration of KCl. Similarly, sucrose-gradient-

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[1] *Abbreviations used in this paper:* CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; CPZ, chlorpromazine; AMP-PNP, adenylyl imidodiphosphate; AMP-PCP, β,γ-methyl-adenosine triphosphate; TEMED, *N,N,N',N'*-tetramethylenediamine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; TnC, rabbit skeletal muscle troponin C; TnI, rabbit skeletal muscle troponin inhibitory subunit I.

purified dyneins obtained from S1-E and S1-K will be referred to as 14S-E or 30S-E and 14S-K or 30S-K dyneins, respectively. Dyneins obtained from a second extraction of P1-E with Tris-EDTA will be designated 14S-E2 and 30S-E2, and those obtained from a second extraction of P1-K with 0.5 M KCl as 14S-K2 and 30S-K2.

In an attempt to optimize conditions for extraction of dyneins with high sensitivity to calmodulin, we have varied the times of extraction of axonemes and of P1-K with 0.5 M KCl. However, no simple relation was found between duration of extraction and degree of calmodulin sensitivity of the dyneins obtained. To avoid uncertainty, the duration of each extraction step will be specified as necessary.

Affinity Chromatography

Partially purified 14S and 30S dyneins, prepared by sucrose density gradient sedimentation, were chromatographed separately over three columns connected in series. Each column was equilibrated with IMT/6 buffer containing 1.5 mM Ca^{++} (column buffer). The columns contained the following resins: column 1, Sepharose 4B (2.5-ml bed volume); column 2, CAPP-Sepharose 4B (2.5-ml bed volume); column 3, calmodulin-Sepharose 4B (10-ml bed volume, 0.6 mg calmodulin/ml). These columns were used to sequentially bind nonspecific aggregates, calmodulin, and proteins with calmodulin binding sites, e.g., dynein ATPases (see Results). The 14S or 30S dynein was applied after a 1:1 dilution with column buffer and addition of Ca^{++} to yield a solution 1.5 mM with respect to this cation. After application of the sample, the columns were washed with 30 ml of column buffer. The columns were then separated and any material bound in a Ca^{++}-dependent manner was eluted from each column with elution buffer (column buffer with 1 mM EGTA replacing the Ca^{++}). The fractions obtained from each column were analyzed as described in Results.

Binding of $[^{35}S]$Calmodulin to Pellet 2

After a second extraction with Tris-EDTA, 0.5 ml of P2-E was added to 0.3 ml IMT buffer (50 mM Tris-HCl, 50 mM imidazole, 7 mM $MgCl_2$, 4 mM EGTA), pH 7.5, 0.1 ml of either 10 mM EGTA or 12.5 mM Ca^{++}, and 0.6 ml of $[^{35}S]$calmodulin (~23,000 cpm) dissolved in H_2O. The mixtures were incubated for 4 min at 25°C and then centrifuged for 20 min at 24,000 *g* at 0°C. 1 ml of the clear supernate was carefully removed and placed in a counting vial. The remaining supernate was decanted and the pellet dissolved in 0.5 ml of 0.5 M NaOH and quantitatively transferred to a counting vial by rinsing of the centrifuge tubes with ACS scintillant (Amersham-Searle Corp., Arlington Heights, Ill.). Radioactivity in the vials was determined in a Tri-Carb spectrometer (Packard Instrument Co., Downers Grove, Ill.) equipped with an automatic external standard and corrected to the same quench as that of 0.5 ml of the $[^{35}S]$calmodulin solution in ACS.

RESULTS

A preliminary study showed that very little ATPase activity was extracted when demembranated axonemes were treated with 0.15 M KCl in IMT/6 buffer for 2 h, and that the pellet P1-K remaining after centrifugation still demonstrated a decrease in turbidity (measured at 350 nm) upon addition of ATP. Addition of Ca^{++} to the supernate (S1-K) caused a small but reproducible ($n > 10$) increase in ATPase activity, whereas addition of Ca^{++} plus 10 µg of bovine brain calmodulin caused up to a 25% increase in ATPase activity. Similar results were obtained with S2-K and P2-K, obtained after a further 2-h extraction of P1-K with 0.3 M KCl in IMT/6 buffer, and with S3-K and P3-K, obtained after extraction of P2-K with 0.5 M KCl in IMT/6 buffer. In these experiments, the ATPase assay was initiated by adding 0.1 ml of Si to 0.9 ml of reaction mixture containing ATP, Ca^{++}, bovine brain calmodulin, and buffer. It was then discovered that if the calmodulin was preincubated with the supernatant fraction, a larger degree of activity enhancement could be obtained, as shown in Table I. Activity enhancement was almost complete in 30 s, and increasing the amount of calmodulin added to the assay from 10 to 20 µg resulted in only a small, but significant, additional increase in ATPase activity. All subsequent experiments containing calmodulin were performed with 10 µg in the assay mixture unless otherwise specified. Calmodulin alone (10 µg) had no ATPase activity.

TABLE I

Effect of Calmodulin on the Supernate Obtained by 0.5 M KCl Extraction of Demembranated Axonemes

Ca^{++}	Calmodulin	Time of preincubation	% ATPase activity
	µg	*min*	
−	0	5	(100)
+	0	5	105
−	10	5	100
+	10	5	155
+	20	5	169
+	10	0	105
+	10	0.5	144
+	10	1.5	150
−	10	3.0	156

Demembranated axonemes were extracted sequentially with 0.15 M KCl, 0.30 M KCl, and 0.50 M KCl (in IMT/6 buffer, pH 7.5) for 2 h, 2 h, and 18 h, as described in the text and in Materials and Methods. The supernate of the 0.5 M KCl extraction, S3-K, had an ATPase activity in the absence of Ca^{++} (i.e., presence of 0.13 mM EGTA) of 0.11 µmol/min per mg (= 100% activity). Each assay contained 92 µg of protein. +*Ca* $^{++}$ indicates the presence of 0.25 mM total Ca^{++} (~0.12 mM free Ca^{++}). Preincubation was at 25°C, as was the ATPase assay, which was terminated after 20 min by addition of trichloroacetic acid as described in Materials and Methods.

In the absence of a large supply of purified *Tetrahymena* calmodulin, we have used homogeneous bovine brain calmodulin for most of these experiments. Because of the similarity in amino acid composition and other physicochemical properties between *Tetrahymena* calmodulin and bovine brain calmodulin (28) and the similarity in their Ca^{++}-dependent activities (28)—i.e., both proteins (*a*) activate partially purified "activator-depleted" bovine brain cyclic nucleotide phosphodiesterase (30), (*b*) form complexes with rabbit skeletal muscle troponin inhibitory subunit (TnI), and (*c*) bind to phenothiazines—it seemed unlikely that there would be any appreciable differences between these two calmodulins in their ability to enhance dynein ATPase activity. To test this asssumption, sufficient *Tetrahymena* calmodulin was prepared to perform the experiments shown in Fig. 1. It can be seen that *Tetrahymena* calmodulin is as effective as bovine brain calmodulin as an activator of the Ca^{++}-dependent ATPase activity of 30S-E dynein. Similar results were obtained with 30S-E2. It appears that bovine brain calmodulin may be a slightly more effective activator of 14S-E dynein ATPase than the *Tetrahymena* calmodulin. Another experiment with 14S-E2 dynein confirmed this observation. Further clarification of the differences between the ATPase stimulating ability of vertebrate and *Tetrahymena* calmodulins, if any, must await the preparation of more *Tetrahymena* calmodulin. In any case, bovine brain calmodulin is at least as effective as *Tetrahymena* calmodulin in enhancing the Ca^{++}-dependent ATPase activities of both 14S-E and 30S-E dyneins, so that studies using the brain protein are likely to yield essentially the same results as would be obtained with *Tetrahymena* calmodulin. ***In what follows, the term "calmodulin" refers to the bovine-brain-derived protein unless otherwise specified.***

Effects of Calmodulin on Dyneins Prepared by Tris-EDTA- and KCl-Extraction Procedures

Table II presents results obtained from studies in which demembranated axonemes were washed in IMT/6 buffer and divided into two equal portions that were treated separately as

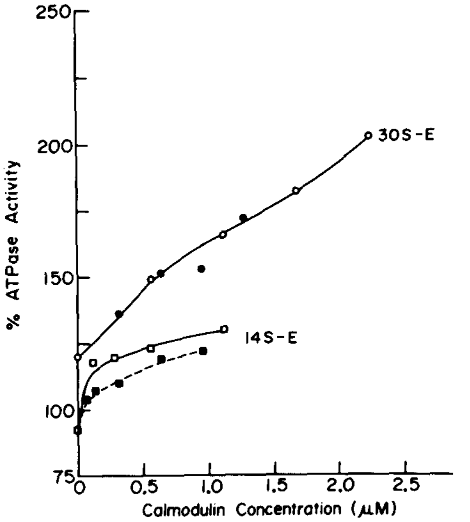

FIGURE 1 Effects of bovine brain calmodulin and of *Tetrahymena* calmodulin on ATPase activity of 14S and 30S dyneins. Demembranated axonemes were extracted with Tris-EDTA as described in Materials and Methods, and 14S-E and 30S-E dyneins were prepared by sucrose density gradient sedimentation. The amount of dynein used in each ATPase activity assay was 13.2 μg of 30S dynein and 11.5 μg of 14S dynein. 100% ATPase activity refers to the activity measured in the absence of added calmodulin, i.e., 0.13 mM EGTA, and was 0.68 and 0.96 μmol/min · mg for the 30S-E and 14S-E dyneins, respectively. All other measurements were made in the presence of 1.25 mM total Ca^{++}, i.e., ~1.1 mM free Ca^{++}. Calmodulin, when present at the concentrations shown on the abscissa, was preincubated with the dynein for 4.0 min at 25°C before the 0.1 ml of 10 mM ATP was added to initiate the ATPase activity assay. Circles, 30S dynein; squares, 14S dynein; open symbols, bovine brain calmodulin; filled symbols, *Tetrahymena* calmodulin.

follows: One portion was extracted with Tris-EDTA, yielding S1-E and P1-E, containing, respectively, 51 and 39% of the original total ATPase activity. The other portion was extracted for 2 h with 0.3 M KCl in IMT/6 buffer and then centrifuged, yielding S1-K and P1-K, containing 25 and 57% of the original ATPase activity. P1-K was then extracted for ~20 h with 0.5 M KCl in IMT/6, yielding S2-K and P2-K, each containing 47% of the activity in P1-K. 3 ml each of S2-K and S1-E were layered onto sucrose density gradients (made up in IMT/6 buffer), and the peak fractions were assayed for ATPase activity in the presence and absence of Ca^{++} and calmodulin. The axonemal ATPase activity was increased by Ca^{++} alone and still more by Ca^{++} plus calmodulin. Almost identical percentage increases were obtained in a second preparation. The crude dynein ATPase obtained by Tris-EDTA extraction (S1-E) was insensitive to addition of Ca^{++} alone but was more sensitive to Ca^{++} in the presence of calmodulin, as was pellet P1-E. When S1-E was resolved on a sucrose gradient, the resulting 30S-E dynein ATPase was more sensitive to Ca^{++} in the presence of calmodulin than was the 14S-E dynein ATPase. However, only a 1.6-fold enhancement of 30S-E dynein ATPase activity was obtained in the presence of 10 μg of calmodulin.

The supernatant (S1-K) and pellet (P1-K) fractions obtained from a 2-h extraction of axonemes with 0.3 M KCl exhibited ATPase activities that were only mildly sensitive to Ca^{++} in the presence of calmodulin (Table II). When P1-K was extracted further with 0.5 M KCl in IMT/6 buffer, the ATPase activity of the resulting supernatant fraction, S2-K, was more sensitive to calmodulin stimulation than any of the fractions obtained from the Tris-EDTA fractionation procedure. Although both the 30S-K2 and 14S-K2 dynein ATPases obtained from sucrose density fractionation of S2-K were more sensitive to calmodulin stimulation than the corresponding fractions obtained from the Tris-EDTA fractionation, it is clear that the major effect of the 0.5 M KCl extraction procedure was to yield a 14S-K2 dynein ATPase that showed a marked enhancement of activity in the presence of Ca^{++} plus calmodulin. In one KCl-extraction experiment, in which the basal ATPase activity of the 14S-K fraction was very low, addition of calmodulin gave a 10-fold enhancement.

TABLE II

Comparison of Calmodulin Effects on Fractions Obtained by Tris-EDTA and KCl Extractions of Ciliary Axonemes

	Total ATPase		Basal activity (no additions)	% Basal activity	
				+ Ca^{++}	+ Ca^{++} and calmodulin
	μmol/min	*%*	*nmol/min · mg*		
Demembranated axonemes	1.5	(100)	89	131	152
A. Tris-EDTA Extraction					
S1-E	0.77	51	202	106	133
P1-E	0.59	39	74	102	141
14S-E	—	—	538	101	129
30S-E	—	—	290	109	161
B. KCl Extraction					
S1-K	0.38	25	89	111	122
P*1-K	0.85*	57*	100*	102*	130*
S2-K	0.40	27	67	107	236
P2-K	0.40	27	139	98	129
14S-K2	—	—	97	104	700
30S-K2	—	—	148	106	193

Demembranated cilia were prepared as described in Materials and Methods and divided into two portions. One portion was extracted with Tris-EDTA for ~18 h and then centrifuged to yield S1-E and P1-E. 3 ml of S1-E were subjected to sucrose density-gradient fractionation, yielding 14S-E and 30S-E. The other portion was extracted for 2 h with 0.3 M KCl in IMT/6, yielding fractions S1-K and P1-K. P1-K was then extracted for ~20 h with 0.5 M KCl in IMT/6, yielding S2-K and P2-K. Because all of P1-K from this preparation was used to prepare S2-K and P2-K, results from a 2-h extraction of another preparation with 0.3 M KCl in IMT/6 are included for completeness, as indicated by an asterisk. 3 ml of S2-K were layered onto a sucrose density gradient and centrifuged at the same time as S1-E. The total units of ATPase activity in each fraction before the density gradient steps are shown, as is the basal activity in the absence of added Ca^{++} (i.e., 0.13 mM EGTA) or calmodulin. In addition, each crude fraction and the peak 14S and 30S fractions from each gradient were assayed with 0.25 mM total Ca^{++} and/or 10 μg calmodulin, as indicated. The protein concentrations during the assays were (in μg/ml): S1-E, 113; P1-E, 251; 14S-E, 9.0; 30S-E, 13.8; S1-K, 208; P1*-K, 144; S2-K, 141; P2-K, 82; 14S-K2, 14.3; 30S-K2, 12.4.

The above experiments were performed with no added Ca^{++} (i.e., 0.13 mM EGTA) or a sufficient excess of Ca^{++} to yield >0.1 mM free Ca^{++} in the assay mixtures. Several experiments were performed to estimate the free Ca^{++} concentration required to produce the full calmodulin-dependent Ca^{++} sensitivity of the ATPase activities. For a preparation of 14S-K dynein (from an 0.5 M KCl extraction of axonemes), full activation (2.6-fold) was obtained at $<1.1 \times 10^{-5}$ M free Ca^{++}. Full activation of the pellet fraction, P1-E, obtained after Tris-EDTA extraction of demembranated axonemes, was evident at $<10^{-5}$ M free Ca^{++}. (Computations, performed with a program provided by Professor C. Tanford of Duke University, included the four Ca^{++} ions bound to the calmodulin.) Although further studies will be necessary to establish the concentration of Ca^{++} required for half-maximal activation of dynein ATPases in the presence of calmodulin, it is clear that the effect occurs in the same range (10^{-6}–10^{-5} M) as required for the Ca^{++} effect on the pellet height response of *Tetrahymena* axonemes (8). It is important to note that the range of minimum Ca^{++} concentration required for maximum ATPase stimulation correlates well with calmodulin's affinity for Ca^{++} (four binding sites, $K_d \cong 1 \times 10^{-6}$ M [40]). It should also be mentioned that there was practically no change in ATPase activity with free Ca^{++} in the range 0.1–2 mM in the presence or absence of calmodulin.

Effect of CPZ on the Ability of Ca^{++} to Cause Enhancement of Dynein ATPase Activity in Presence of Calmodulin

In the presence of Ca^{++}, calmodulin has a high affinity for CPZ (32, 49), a fact that serves as the basis for the use of an analogue of CPZ (i.e., CAPP) as a ligand for the affinity-column purification of calmodulin (27) (see Materials and Methods). CPZ has been shown to inhibit a number of Ca^{++}-calmodulin-activated enzymatic activities (44, 49), and in our earlier report (28), it was noted that CPZ partially inhibited the ATPase activities of axonemes and of 14S and 30S dyneins. It was therefore of interest to ascertain whether low concentrations of CPZ might negate the activity enhancing effects of calmodulin in the presence of calcium. In the experiment shown in Table III, axonemes were extracted for 40 min with 0.3 M KCl, followed by 1.2 h with 0.5 M KCl, yielding an S2-K that was then subjected to sucrose density gradient sedimentation. Addition of calmodulin in the absence of added Ca^{++} (0.13 mM EGTA; $\sim10^{-8}$ M free Ca^{++}) caused a slight increase in ATPase activity of both 14S-K2 and 30S-K2 dynein ATPases, as did addition of Ca^{++} alone. In the presence of both Ca^{++} and calmodulin, the 30S-K2 dynein ATPase activity was stimulated ~1.7-fold, and that of 14S-K2 dynein ~5.9-fold. Addition of 6.3 μM CPZ largely prevented the rise in ATPase activity of 14S-K2 dynein ATPase in the presence of Ca^{++} and calmodulin (see Table III) without affecting the basal activity in the absence of added calmodulin. This also appeared to be true for the 30S-K2 dynein ATPase, but the much smaller degree of ATPase activity enhancement makes interpretation of the data less certain.

TABLE III

Effect of Calmodulin, CPZ, and Ca^{++} on 14S and 30S Dyneins from Axonemes Extracted with 0.5 M KCl

Additions			% Basal ATPase activity	
Ca^{++}	Calmodulin	Chlorpromazine	30S-K2 dynein	14S-K2 dynein
–	–	–	(100)	(100)
–	+	–	127	118
+	–	–	114	104
+	+	–	165	588
+	–	+	120	103
+	+	+	134	129

Demembranated axonemes were extracted for 40 min with 0.3 M KCl in IMT/6 buffer and the pellet (P1-K) obtained by centrifugation was then extracted with 0.5 M KCl in IMT/6 buffer for ~18 h as described in Materials and Methods, yielding P2-K and S2-K. Dyneins in supernate S2-K were resolved by sucrose gradient sedimentation. The peak fractions of the 14S-K2 and 30S-K2 fractions had ATPase activities of 182 and 127 nmol/min·mg, respectively, taken as 100% in the Table. The protein concentrations during the ATPase assays were 16.8 and 16.6 μg/ml for the 14S-K2 and 30S-K2 dyneins, respectively. Ca^{++}, calmodulin, and CPZ when present, were 0.25 mM total (~0.12 mM free), 10 μg, and 6.3 μM, respectively. Reactions were initiated by the addition of 0.1 ml of 10 mM ATP to the assay mixtures 4 min after the dyneins had been added to mixtures containing buffer and the other additions as indicated.

Inhibition of ATPase Activation by Adenine Nucleotides

It was noted above (see text pertaining to Table I) that the effect of calmodulin in causing enhancement of the ATPase activity of crude dynein required a brief preincubation of the crude dynein fraction with calmodulin in the absence of ATP. In those experiments, not only was a crude dynein used but the ATP concentration was 1 mM, the standard concentration used in our ATPase assay procedure. It was therefore of interest to ascertain whether the prevention of the Ca^{++}- and calmodulin-dependent enhancement of ATPase activity occurred with sucrose-density-purified dyneins, whether it was a function of ATP concentration, and whether AMP-PNP and AMP-PCP, nonhydrolyzable analogues of ATP that have proven useful in probing the properties of dynein ATPases (10, 38), could also prevent the enhancing effect of calmodulin.

Exp. *I* of Table IV shows the results of an experiment in which 30S-K and 14S-K dyneins, prepared by extraction with

TABLE IV

Effect of Presence of ATP, AMP-PNP, or AMP-PCP during Preincubation with Calmodulin on Enhancement of Dynein ATPase Activity

	Additions		% Basal ATPase activity			
			30S-K2 dynein		14S-K2 dynein	
Exp.	Calmodulin	Nucleotide	Start*	End*	Start	End
I	–	—	(100)		(100)	
	+	—	132		320	
	+	ATP, 10^{-4} M	84	136	132	300
	+	AMP-PNP, 10^{-4} M	125	150	248	291
	+	AMP-PCP, 10^{-4} M	82	76	207	194
			P1-K dynein		14S-K dynein	
II	–	—	(100)		(100)	
	+	—	154		438	
	+	ATP, 1.1×10^{-4} M	121	152	175	457
	+	ATP, 2.2×10^{-5} M	148	151	232	435
	+	ATP, 5.6×10^{-6} M	158	149	306	418
	+	AMP-PNP, 2.2×10^{-4} M	117	132	318	418

Exp. I was performed on the same cilia preparation described in the legend to Table III. Samples under *start* were preincubated for 240 s in the presence of 0.13 mM free Ca^{++}, calmodulin, and the indicated nucleotide. Control assays (under *end*) received the nucleotide after 260 s of incubation. The ATPase assay was then initiated by adding 0.1 ml of 10 mM ATP to both sets of assay mixtures (thus bringing the final volume to 1 ml). Exp. II was performed on a different preparation of cilia that was extracted for 3.8 h with 0.5 M KCl in IMT/6 buffer. After centrifugation, the pellet, P1-K, was resuspended in IMT/6 and assayed as described for exp. I, as was the 14S-K dynein obtained by sucrose density sedimentation of the supernate, S1-K. For exp. I, 100% ATPase activity of the 30S-K2 and 14S-K2 dyneins was 226 and 213 nmol/min·mg, respectively, and the protein concentrations during the assays were 17.5 and 14.0 μg/ml, respectively. For exp. II, 100% ATPase activity was 80.5 nmol/min·mg for P1-K and 79.9 nmol/min·mg for the 14S-K dynein, and the protein concentrations during the ATPase assays were 155 and 12.8 μg/ml, respectively.

* *Start*, nucleotide added at start of preincubation interval; *end*, nucleotide added at end of preincubation interval.

0.5 M KCl, were preincubated for 4 min in the presence of 0.1 mM ATP, AMP-PNP, or AMP-PCP. As controls, the same concentrations of these nucleotides were added at the end of the 4-min preincubation period, just before addition of 1 mM ATP for the 20-min ATPase assay. With no additions to the reaction mixtures containing 14S-K dynein and calmodulin (plus Ca^{++}), a 3.2-fold enhancement of ATPase activity occurred. Similar results were obtained when 0.1 mM ATP or AMP-PNP was added at the end of the preincubation period. However, when the same concentration of ATP or AMP-PNP was present at the start of the preincubation, only a 1.3-fold (for ATP) or a 2.5-fold (for AMP-PNP) activation was obtained. Hence, 0.1 mM ATP almost completely prevented the stimulation effect of calmodulin on 14S-K dynein ATPase. Though 0.1 mM AMP-PNP was less effective, it nevertheless prevented some of the Ca^{++}-calmodulin-induced activation of ATPase activity. AMP-PCP is a stronger inhibitor of dynein ATPase than is AMP-PNP (10). This is evident in exp. *I* of Table IV. The inhibition of 14S-K dynein activity by 0.1 mM AMP-PCP was about the same whether the AMP-PCP was added at the beginning or end of the preincubation period with calmodulin, suggesting that AMP-PCP did not prevent Ca^{++}-calmodulin ATPase-activity enhancement but acted only as an inhibitor of ATPase activity. As shown in Table IV, similar results were obtained for 30S-K dynein, but because the enhancement effect observed was much smaller than for 14S-K dynein, conclusions similar to those presented above must be regarded as tentative.

Exp. *II* of Table IV shows that considerable inhibition of the stimulatory effect of Ca^{++}-calmodulin on 14S-K dynein ATPase can be obtained by the presence of an ATP concentration as low as 5.6 μM during preincubation. Equivalent inhibition of 14S-K ATPase activation by Ca^{++}-calmodulin was obtained with 1.1×10^{-4} M ATP and 2.2×10^{-4} M AMP-PNP. These results also demonstrate that ATP can partially prevent the Ca^{++}-calmodulin enhancement of ATPase activity in the pellet, P1-K, obtained from a 3.8-h extraction of axonemes with KCl. However, it appears that a much higher concentration of ATP is required for prevention of the Ca^{++}-calmodulin stimulatory effects on the pellet ATPase. Again this statement must be regarded as tentative because of the small degree of activity stimulation observed. Based on these results, all subsequent assays employed a 4-min preincubation of the calmodulin with dynein before initiation of the assay, providing ample time for the calmodulin effect to occur.

Sensitivity of 14S and 30S Dyneins to Ca^{++}-calmodulin-induced Enhancement of ATPase Activity

In the experiments so far described, bovine brain calmodulin was used at a concentration of 10 μg/assay, with one exception (Table I), in which 20 μg yielded only a slight increase in enhancement over that obtained with 10 μg. In that experiment, however, the dyneins were not preincubated with the calmodulin before addition of ATP. Figs. 1 and 2 show the results of detailed analyses of the effects of calmodulin concentration on dynein ATPase activity. The effect of added calmodulin on the Ca^{++}-dependent ATPase activity of 14S-E dynein differs from that of 30S-E dynein (Fig. 1). The ATPase activity of 30S-E dynein rises almost linearly with increasing calmodulin concentration up to at least 2.2 μM calmodulin. On the other hand, 14S-E dynein ATPase activity rises sharply at very low concentrations of calmodulin and then only slowly as the calmodulin concentration increases above 0.2 μM. Identical results were obtained with 14S-E and 30S-E dyneins obtained from a different preparation of axonemes. The response of 14S-K2 dynein, obtained from extraction of axonemes by 0.5 M KCl for 6 h followed by a second extraction with 0.5 M KCl for 21 h is shown in Fig. 2. The 14S-K2 dynein ATPase activity was stimulated more than eightfold by calmodulin (in the presence of Ca^{++}), half-maximal enhancement occurring, as with 14S-E dynein, at ~0.1 μM calmodulin. The Ca^{++}-dependent ATPase activity of 30S-K2 dynein was similar to that of 30S-E, i.e., a slowly increasing activity with increasing calmodulin concentration that appeared to saturate at ~2.2 μM (Fig. 2). Thus 14S dynein, whether prepared by Tris-EDTA or KCl extraction, has a Ca^{++}-dependent ATPase activity that is sensitive to a much lower concentration of calmodulin than is the activity of 30S dynein.

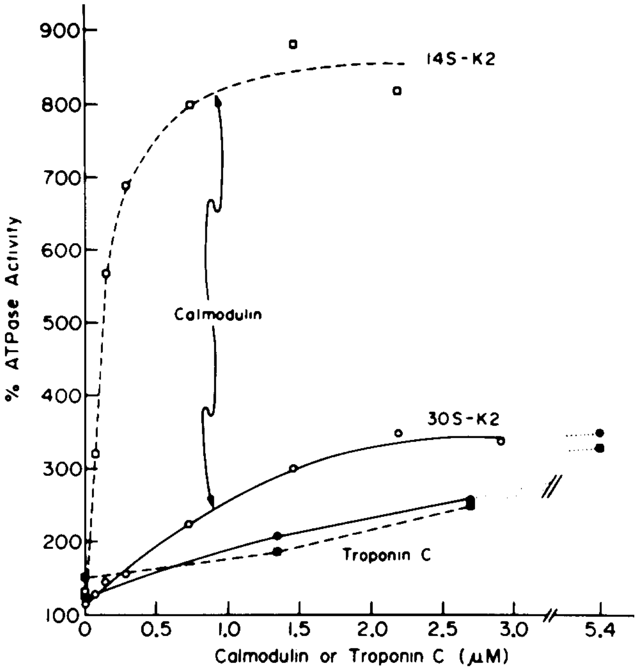

FIGURE 2 Effects of bovine brain calmodulin and of TnC on ATPase activities of 14S and 30S dyneins prepared by KCl extraction. Demembranated axonemes were extracted for 6 h with 0.5 M KCl in IMT/6 buffer, pH 7.5, and then centrifuged, yielding P1-K and S1-K. P1-K was again extracted in the same buffer for 20 h and centrifuged to yield P2-K and S2-K. Dyneins (14S-K2 and 30S-K2) were prepared from S2-K by sucrose density gradient sedimentation as described in Materials and Methods. Each assay mixture contained 18.5 μg of 30S-K2 or 8.5 μg of 14S-K2. 100% ATPase activity, measured in the absence of added Ca^{++}, was 0.062 and 0.26 μmol/min·mg for 30S-K2 and 14S-K2, respectively. All other measurements were made in the presence of 1.25 mM total Ca^{++}. Open symbols, bovine brain calmodulin; filled symbols, TnC. Solid lines, 30S-K2; dashed lines, 14S-K2. Both calmodulin and TnC were preincubated for 4.0 min with the dynein before the ATPase assay was initiated by addition of ATP.

The results presented above show that 14S-K and, to a lesser extent, 30S-K dyneins have Ca^{++}-dependent ATPases that are more sensitive to stimulation by calmodulin than those obtained by EDTA extraction. It was therefore of interest to examine the effect of KCl treatment on 30S and 14S dyneins that had been prepared from Tris-EDTA-extracted axonemes. As can be seen in Table V, incubation with 0.5 M KCl caused an increase in the basal ATPase activity for both dyneins. However, a concomitant increase in activatability of ATPase activities was not observed. It appears, therefore, that conditions during extraction from the axonemes (and possibly during subsequent sedimentation through the sucrose density gradient), rather than the effect of KCl on the extracted dyneins, determine the subsequent sensitivity of Ca^{++}-dependent ATPase activity to calmodulin.

Effect of TnC on Dynein ATPase Activities

Bovine brain calmodulin has considerable structural homology with the Ca^{++}-regulatory protein of actomyosin ATPase, TnC (22, 42, 46, 47), interacts Ca^{++}-dependently with the inhibitory subunit of the actomyosin regulatory complex, TnI, and can substitute for TnC in conferring Ca^{++}-sensitivity on actomyosin ATPase (1). It was therefore of interest to ascertain whether TnC could replace calmodulin in conferring Ca^{++}-dependent activation on dynein ATPase activities. As shown in Fig. 2, TnC activated both 14S-K2 and 30S-K2 ATPases to a limited extent. The activation was less than that achieved with similar concentrations of calmodulin, and TnC stimulated the activity of 14S-K2 dynein much less than it did 30S-K2 dynein. This difference in sensitivity to TnC between 14S and 30S dyneins was also observed with dyneins prepared by Tris-EDTA extraction of axonemes (Fig. 3). Fig. 2 further shows that although addition of 1.5 μM calmodulin to the assay system increased the Ca^{++}-dependent ATPase activities of 14S-K2 and 30S-K2 dyneins eightfold and threefold, respectively, the same concentration of TnC yielded less than a twofold increase of the ATPase activity of either dynein. At the highest concentration of TnC studied (5.4 μM), the same increase in ATPase activity (~3.2-fold) was obtained for 30S-K2 dynein as was obtained with calmodulin, but the activation of 14S-K2 by this amount of TnC was much less than that caused by calmodulin (Fig. 2). Thus the ATPase activity of 14S dynein is much less responsive to Ca^{++}-dependent stimulation by TnC than is the ATPase activity of 30S dynein ATPase, regardless of whether the dyneins are prepared by KCl or Tris-EDTA extraction of axonemes. In preliminary experiments, we have found 0.4 μM TnC to have no effect on the ATPase activities of either P1-K or P1-E.

Affinity Chromatography of 14S and 30S Dyneins

The ability of calmodulin to confer Ca^{++}-sensitivity on 14S and 30S dynein ATPases implies the presence of a calmodulin-binding site(s) on these enzymes. Direct evidence for the presence of such a binding site(s) was sought by subjecting the dyneins to Ca^{++}-dependent affinity chromatography on a calmodulin-Sepharose 4B column. In an effort to remove any aggregates that might be present, and to remove any endogenous calmodulin present in the samples, the material to be analyzed (pooled 30S-E or 14S-E fractions from Tris-EDTA-extracted axonemes resolved by sucrose density gradient sedimentation) was passed sequentially through a Sepharose 4B column and a CAPP-Sepharose 4B column connected serially to and mounted vertically above the calmodulin-Sepharose 4B column (see Materials and Methods). The columns were washed with a Ca^{++}-containing buffer, and then eluted separately with a buffer containing EGTA to release any proteins that bound in a Ca^{++}-dependent manner. The results of one such experiment are presented in Fig. 4; essentially identical results were obtained with 30S-E and 14S-E dyneins from a different preparation of Tris-EDTA-extracted axonemes. Analysis of the material that passed unretarded through the chromotographic analysis described above revealed little ATPase activity or protein to be present (see Figs. 4 and 5). For both the 14S and 30S dynein affinity-chromatography separations, EGTA elution of the Sepharose 4B column also yielded an eluant virtually devoid of protein and ATPase activity, suggesting that few if any large aggregates had been nonspecifically adsorbed to the Sepharose 4B resin. Similarly, the EGTA eluant from the CAPP-Sepharose 4B column contained very little protein and had no ATPase activity (Fig. 4). Examination

TABLE V

Effect of Incubation of 30S-E and 14S-E Dyneins with 0.5 M KCl on Subsequent Sensitivity of Ca^{++}-dependent ATPase to Calmodulin

	14S dynein ATPase			30S dynein ATPase		
	+	−	+/−	+	−	+/−
	μmols/ min· mg			μmols/ min· mg		
Control	0.99	0.76	1.30	0.96	0.84	1.14
Incubated with KCl	1.01	0.87	1.16	1.43	1.36	1.05
Value expected for dyneins prepared by KCl extraction			(3-10)			(1.5-2)

30S and 14S dyneins were prepared from Tris-EDTA-extracted axonemes, as described in Materials and Methods. About 45 μg of each were incubated at 25°C for 2.3 h in IMT/6 (pH 7.5) buffer containing no KCl (*control*) or 0.5 M KCl. 0.2-ml samples were then assayed for ATPase activity with 1.25 mM total Ca^{++} in the presence (+) and absence (−) of 10 μg of bovine brain calmodulin with a 4-min preincubation before addition of ATP. The concentration of KCl (when present) during the 20-min ATPase assay was 0.1 M.

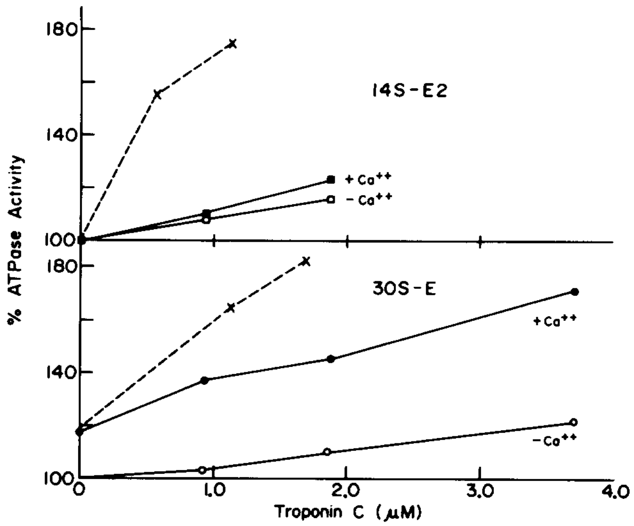

FIGURE 3 Ca^{++}-dependent effect of TnC on ATPase activity of 30S and 14S dynein. Demembranated axonemes were extracted for 22 h with Tris-EDTA as described in Materials and Methods. Centrifugation yielded P1-E and S1-E. P1-E was reextracted for 23 h with Tris-EDTA and then centrifuged to yield P2-E and S2-E. 14S-E and 30S-E dyneins were obtained from S1-E by sucrose density gradient sedimentation, as were 14S-E2 and 30S-E2 from S2-E. Open symbols, no added Ca^{++}; filled symbols, 1.25 mM total Ca^{++}. Circles, 30S dynein; squares, 14S-E2 dynein. 100% ATPase activity, measured in the absence of added Ca^{++} or of TnC, was 0.72 and 2.26 μmol/min·mg for 30S and 14S-E2 dyneins, respectively. The dashed lines show the effect of calmodulin added at the concentration indicated on the abscissa.

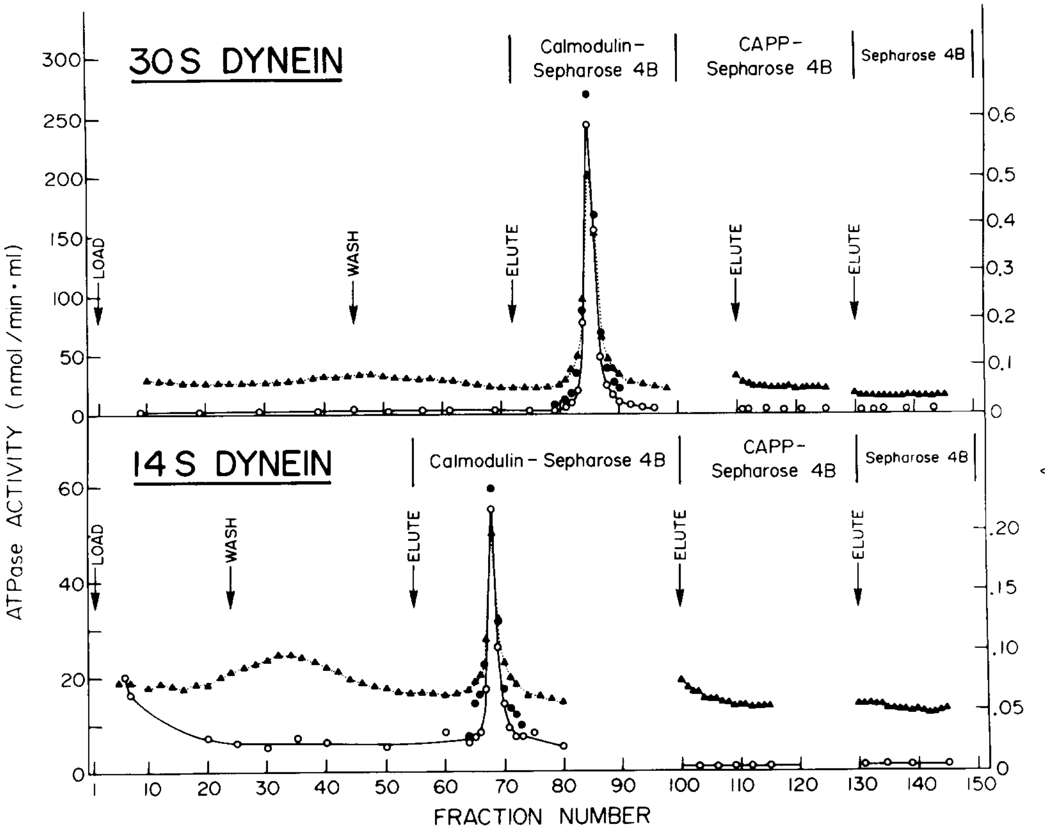

FIGURE 4 Serial Sepharose 4B/CAPP-Sepharose 4B/Calmodulin-Sepharose 4B affinity chromatography of 14S and 30S dyneins prepared from Tris-EDTA-extracted axonemes. 9 ml of pooled 14S dynein (1.0 mg protein), lower panel, and 11.8 ml of pooled 30S dynein (1.9 mg protein), upper panel, were diluted 1:1 with IMT/6 + 1.5 mM Ca^{++}, pH 7.5, and applied onto a column of Sepharose 4B that was connected to a CAPP-Sepharose 4B column and finally to a column of calmodulin-Sepharose 4B, as described in Materials and Methods. After sample application was complete, the columns were washed with ~30 ml IMT/6 (pH 7.5) buffer containing 1.5 mM Ca^{++}. The columns were then disconnected and eluted separately with buffer containing 1.0 mM EGTA instead of the calcium. The A_{280} (▲) of each fraction was determined and aliquots were taken from selected fractions for assay of ATPase activity (1.25 mM total Ca^{++}) in the absence of added calmodulin (○) or after addition of 10 μg of bovine brain calmodulin (●). For further details, see text.

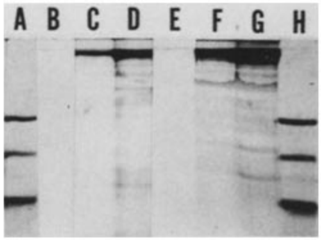

FIGURE 5 Analysis of fractions from affinity chromatography of 14S and 30S dyneins by gel electrophoresis. Fractions from the experiments shown in Fig. 4 were subjected to slab gel electrophoresis on SDS-7.5% polyacrylamide gels. Lanes: *A* and *H*, 4 μg each of M_r standards (phosphorylase *b*, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; and carbonic anhydrase, 30,000); *B*, 0.5 ml of fraction 30 of the 14S dynein affinity-column separation; *C* and *D*, 2.5 and 5.0 μg, respectively, of the 14S dynein eluted from the calmodulin-Sepharose 4B column; *E*, 0.5 ml of fraction 50 of the 30S dynein affinity-chromatography separation; *F* and *G*, 2.5 and 5.0 μg, respectively, of the 30S dynein eluted from the calmodulin-Sepharose 4B column. The gel was electrophoresed until the dye band was ~1 cm from the bottom of the gel.

of the CAPP-Sepharose 4B EGTA-eluant fractions by alkaline urea-PAGE did not reveal the presence of calmodulin, but the presence of small amounts of calmodulin below the limits of sensitivity of the analysis cannot be excluded. Recent experiments using [^{35}S]*Tetrahymena* calmodulin support this conclusion. Over 99% of the [^{35}S]calmodulin added to the material applied to an identical set of serial affinity columns was retained by the CAPP-Sepharose 4B column in the presence of Ca^{++}.

When the calmodulin-Sepharose 4B column was eluted with the EGTA-containing buffer, a single sharp peak of protein and of basal ATPase activity was eluted (Fig. 4) demonstrating the presence of a Ca^{++}-dependent binding site(s) for calmodulin on both 14S and 30S dyneins. In one experiment, the pooled fractions from the calmodulin-Sepharose 4B column of 30S dynein were resedimented in a sucrose density gradient to ascertain whether the eluted ATPase was still a "30S" dynein. Most of the ATPase activity sedimented as 30S dynein, with only a small amount of material—thought to be aggregates—sedimenting at a greater S value.

Fractions containing the calmodulin-Sepharose 4B affinity column-purified dyneins were also assayed for ATPase activity in the presence of 10 μg of bovine brain calmodulin and Ca^{++}. It can be seen (Fig. 4) that, unlike the 14S or 30S dyneins that had been loaded onto the column, which had calmodulin stimulation indices (+ calmodulin/− calmodulin) of 1.2 and 1.9, respectively, the ATPase activities of the eluted dyneins in this experiment were only marginally sensitive to the addition of 10 μg of calmodulin.

However, in another affinity-chromatography experiment with 14S-E and 30S-E dyneins, sensitivity to 10 μg calmodulin was still apparent, and it was confirmed that by adding more calmodulin (>10 μg per reaction mixture) to the 30S dynein eluted from the calmodulin-Sepharose 4B column, a larger degree of (Ca^{++}-dependent) enhancement of the ATPase activity was achievable. In one experiment with 30S-K dynein eluted from the calmodulin-Sepharose 4B, an ATPase activity ratio of 1.6 (with 10 μg calmodulin per assay) was observed. Thus, although there may be a variable reduction in sensitivity to added calmodulin—perhaps because of the variable loss of other regulatory components—both the 14S and 30S dynein ATPases that are eluted from calmodulin-Sepharose 4B columns are still stimulated by the addition of calmodulin. The presence of other regulatory components is also suggested by the variable sensitivity of dyneins prepared by several methods to calmodulin stimulation of ATPase activity (e.g., 14S-K1 vs. 14S-K2 or EDTA- vs. KCl-extracted material). The reasons for this variable, partial loss of sensitivity to calmodulin stimulation of ATPase activity are the subject of current investigations.

Analysis of Affinity-column-purified Dyneins by PAGE

Portions of the fractions from the affinity-chromatography separation shown in Fig. 4 were subjected to electrophoresis in 7.5% SDS polyacrylamide gels. Fig. 5 shows that, in agreement with the very low A_{280} levels, there were no discernible protein bands in the unretarded fractions from either column. It also shows that a majority of the Ca^{++}-dependently bound 14S and 30S dyneins consisted of high molecular weight components. At these high loads, at least 10 intermediate and low molecular weight components were observed in addition to the high molecular weight components of both the 14S and 30S dyneins. Because the high molecular weight components predominated, there is some question as to whether these intermediate and low molecular weight bands are stoichiometric components of the dyneins, minor contaminants, or limited degradation products. To obtain further information about the high molecular weight bands, which scarcely penetrate the 7.5% gel in the time required for the dye front to reach 1 cm from the anodal end of the gel, suitable aliquots were analyzed on the same gels electrophoresed for twice this time (Fig. 6). It can be seen that two major (~260,000 and 253,000 M_r) and one minor (~270,000 M_r) components compose the bulk of the isolated 14S dynein. The 30S dynein was composed of one major component (~270,000 M_r), possibly corresponding to the minor band of 14S dynein, and a lesser amount of a 246,000-dalton component that is not found in the 14S dynein. It is also of interest that 14S-E2 and 30S-E2 dyneins appear to consist of components identical to their E1 counterparts (Fig. 6). Because these M_r values were obtained by extrapolation of the graph of log molecular weight vs. migration distance for the standards, the largest of which was myosin (200,000 mol wt), the molecular weights estimated above are clearly approximations. The two main bands of 14S dynein observed here probably correspond to the 375,000- and 358,000-dalton bands of 14S dynein found by Hayashi and Takahashi (25) analyzed on 3.5% gels, and to the single (broad) B band (~520,000 daltons) found by Mabuchi and Shimizu (33), using 3% gels. The main band of the 30S dynein found here corresponds to the main band (~560,000 daltons) found by Hayashi and Takahashi (25) and to the band (~560,000 daltons) found by Mabuchi and Shimizu (33). It is not clear whether our 246,000-M_r component corresponds to any of the bands observed by other workers. It seems likely from the present results as well as those of the earlier workers that 14S dynein as composed largely of two high molecular weight polypeptides present in roughly equal amounts, whereas 30S dynein contains predominantly a component of slightly higher molecular weight than either of the two major components of 14S dynein. According to Hayashi and Takahashi (25), the 30S dynein band may itself consist of three closely spaced bands. The difference in results obtained by various workers (25, 33, 34; this report, Fig. 6) suggest that much remains to be learned about the polypeptide components of the dyneins of *Tetrahymena* cilia.

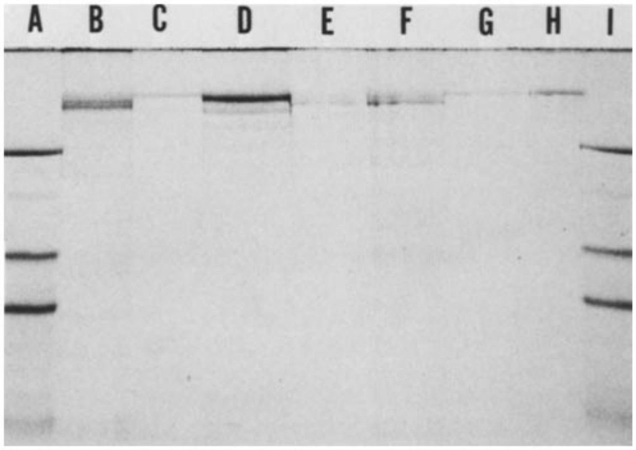

FIGURE 6 Electrophoretic analysis of affinity-purified dyneins. Electrophoresis was performed on SDS-7.5% polyacrylamide gels for a time twice that required for the dye band to reach the bottom of the gel. Lanes: *A* and *I*, 4 μg each of M_r standards (myosin, 200,000; β-galactosidase, 116,000; phosphorylase *b*, 94,000; bovine serum albumin, 68,000; and ovalbumin, 43,000); *B*, 4 μg of the affinity-purified 14S dynein (fraction 68, Fig. 4); *C* and *D*, 1.5 and 6 μg, respectively, of affinity-purified 30S dynein (fraction 85, Fig. 4); *E* and *F*, 1.5 and 3 μg, respectively, of a preparation of 14S-E2 dynein; *G* and *H*, 1 and 2 μg, respectively, of 30S-E2 dynein obtained from the same preparation as that used for isolation of the 14S-E2 dynein used in lanes *E* and *F*. For details of the preparation of 14S-E2 and 30S-E2 dyneins, see Materials and Methods.

Calmodulin Binding to Tris-EDTA-extracted Axonemes

We have earlier shown (3) that even after a second extraction with Tris-EDTA, a residual ATPase activity remains associated with the twice-extracted axonemal pellet (P2-E) and that this residual ATPase activity differs in several ways from that of extracted dyneins (3, 5, 9). Having established the presence of binding sites for calmodulin in 14S and 30S dyneins, we found it of interest to ascertain whether any calmodulin binding sites remained in twice-extracted axonemes. An experiment was therefore performed in which P2-E (2 mg of protein from the same preparation of cilia as used in Fig. 2) was added to tubes containing 0.75 mM EGTA, or 0.75 mM free Ca^{++}, and [^{35}S]calmodulin (prepared as described in Materials and Methods). Quadruplicate samples with EGTA or Ca^{++} were centrifuged and the counts per minute remaining in the supernate and in the pellet were measured (see Materials and Methods). The amount of added [^{35}S]calmodulin (sum of counts per minute in pellet plus supernate) was 22,750 ± 940 cpm (SD; *n*

= 8) and its specific activity was estimated to be 1.3×10^5 cpm/μg (see Materials and Methods). The pellets obtained from the tubes with EGTA and Ca^{++} contained 4746 ± 478 cpm (n = 4) and 10,910 ± 530 cpm (n = 4), respectively, thus demonstrating the presence of a Ca^{++}-dependent calmodulin binding site(s) in a pellet from which a majority of the extractable dynein had been removed. In these experiments no markers were included to allow computation of the water space of the pellet, but from subsequent studies (unpublished data) we can safely assume that it was <0.05 ml. Thus even if one corrects the counts per minute in the pellets obtained in the absence of Ca^{++} for the maximum amount of calmodulin remaining in the water space (~1,800 cpm), there still remains ~300 cpm of ^{35}S-calmodulin that bound to the pellets in the absence of Ca^{++}. Despite this Ca^{++}-independent binding ~6,160 cpm were bound to P2-E in a Ca^{++}-dependent manner, corresponding to ~25 μg [^{35}S]calmodulin per gram of pellet. Similar results were obtained when this experiment was performed on the same pellet after storage for 8 d at 4°C in IMT/6, pH 7.5, indicating that these Ca^{++}-dependent binding sites are stable for at least this period of time.

DISCUSSION

The present studies establish that 14S and 30S dyneins and twice-extracted axonemes contain Ca^{++}-dependent binding sites for calmodulin and that addition of calmodulin confers Ca^{++}-sensitivity on the dynein ATPases obtained using the extraction procedures detailed herein (see Materials and Methods and Results). Doughty (17) has also reported that crude dynein prepared by 0.5 M KCl extraction of *Paramecium* cilia is sensitive to Ca^{++} in ATPase assays performed with 1 mM Mg^{++} and 1 mM ATP. In his studies, conducted in the absence of EGTA, low concentrations of Ca^{++} caused marked inhibition of the ATPase activity. However, at Ca^{++} concentrations >8 μM, he observed an increase in ATPase activity up to the level observed in the absence of added Ca^{++}. Upon purification of the crude dynein by gel filtration chromatography on Sepharose 4B, three dynein fractions were obtained. The ATPase activity of dynein I was increased ~1.6-fold by low concentrations of Ca^{++}, and then reduced towards the initial, basal level as the [Ca^{++}] exceeded ~8 μM. That of dynein II increased ~1.3-fold as the [Ca^{++}] was increased up to ~0.5 mM, whereas that of dynein III was inhibited ~50% as the [Ca^{++}] was increased to ~8 μM and then increased towards the initial, basal level, observed in the absence of added Ca^{++}, upon further increases in [Ca^{++}] (17).

At the free [Ca^{++}] used in the experiments presented here, we have always observed only a slight Ca^{++}-induced increase in dynein ATPase activity, whether examining dynein *in situ*, the original demembranated axonemes, any of the extracted pellet fractions, crude dynein, or partially purified 30S dynein. The ATPase activity of 14S dynein was occasionally slightly inhibited by Ca^{++} in the absence of added calmodulin (see, for example, Fig. 1). However, appreciable Ca^{++}-dependent stimulation of dynein ATPase activities (measured in the presence of 2.4 mM Mg^{++} and 1 mM ATP) was evident only with added calmodulin. Whether this difference is attributable to: (*a*) a difference between the cilia of these closely related species, (*b*) the higher [Mg^{++}] used in the present experiments, (*c*) the presence of EGTA in our studies, or (*d*) the method of deciliation/demembranation employed remains to be determined.

Calmodulin Induction of Dynein ATPase Ca^{++}-sensitivity and the Nature of the Calmodulin-binding Sites

Examination of the differential abilities of calmodulin and the closely related calcium-binding protein TnC to activate the dynein ATPase activities has proved useful in demonstrating the specificity of calmodulin in this system. The Ca^{++}-dependent ATPase activity of 14S dynein is stimulated by a lower concentration of calmodulin than is required for stimulation of 30S dynein, regardless of whether the dyneins are prepared by Tris-EDTA or by KCl extraction of the axonemes. TnC also stimulates the Ca^{++}-dependent ATPase activities of the dyneins, but to a limited extent. It stimulates 30S-E dynein more effectively than 14S-E (Fig. 3). The activities of 14S-K2 and 30S-K2 dyneins are stimulated approximately equally (Fig. 2), but the concentration of TnC required for half-maximal saturation of 30S-K2 dynein ATPase is much less than that for 14S-K2 dynein ATPase. Thus, whereas calmodulin confers calcium sensitivity to both 14S and 30S dynein ATPase, TnC confers significant sensitivity only to 30S dynein ATPase. This suggests that the calmodulin-binding sites on the two dyneins may differ.

Despite numerous differences, there are a large number of similarities between the kinetic properties of dynein ATPase and myosin ATPase (see reference 13 for review). The only documented role for calmodulin in actomyosin regulation in vivo is through the activation of myosin light-chain kinase (16, 24, 36). Murofushi (35) reported that three cyclic AMP–independent protein kinase activities could be found in *Tetrahymena* axonemes. Although these have not yet been systematically examined for possible Ca^{++}-calmodulin sensitivity, it is possible that calmodulin may regulate a kinase that modulates the activity of one or both of the dyneins.[2]

The finding that TnC is an effective activator of 30S dynein ATPase raises the possibility that although calmodulin acts as the endogenous activator of 14S dynein ATPase, an as yet undetected TnC-like protein is the endogenous activator of 30S dynein ATPase. Recent studies by Gitelman and Witman (21) indicate that although calmodulin is present in *Chlamydomonas* flagella, it is not found associated with the isolated dyneins. In addition, the flagella of *Chlamydomonas* contain a TnC-like Ca^{++}-binding protein that does not appear to be calmodulin.[3]

The effects of ATP and ATP analogues on calmodulin activation of dynein ATPases, presented in Results, provide some insight into the mechanism through which activation occurs. The presence of a high-affinity ATP-binding site on 30S dynein was previously deduced from studies on the ability of ATP to prevent activation of ATPase activity by *bis*(4-fluoro-3-nitrophenyl)sulfone (10). It was also demonstrated in that report that the nonhydrolyzable ATP analogue AMP-PNP was less effective than ATP as a preventive reagent, whereas AMP-PCP, a stronger inhibitor of 30S dynein ATPase activity

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[2] The ability of extracts of *Tetrahymena* axonemes to phosphorylate casein (using an assay similar to that described by Murofushi [35]) was not altered by addition of calcium plus calmodulin (unpublished data). We have not, however, attempted purification of the kinase activities by column chromatography or examined the individual kinases for possible calcium-calmodulin regulation.

[3] VanEldik, L. J., G. Piperno, and D. M. Watterson. Similarities and dissimilarities between calmodulin and a *Chlamydomonas* flagellar protein. *Proc. Natl. Acad. Sci. U. S. A.* In press.

than AMP-PNP, did not appear to prevent activation. The discovery here that low concentrations of ATP (~5 μM) and moderate concentrations of AMP-PNP (~200 μM) partially prevent Ca^{++}-calmodulin stimulation of dynein ATPase activity, whereas AMP-PCP does not, (*a*) correlates with our previous results (10), (*b*) suggests that 14S dynein also has a high-affinity ATP-binding site, and (*c*) indicates a close connection between the calmodulin-binding site and the high-affinity ATP-binding site.

Although the interaction of calmodulin with dynein that results in Ca^{++}-dependent activation of dynein ATPase activities was determined to be rapid, it does not appear from the data presented here that activation occurs through a simple, diffusion-limited process. Clearly, detailed kinetic analyses will be required to further clarify this point.

Affinity Chromatography and Gel Electrophoresis of 14S and 30S Dyneins

Calcium-dependent affinity chromatography on calmodulin-Sepharose 4B conjugates (45) is one of the major tests used to demonstrate calmodulin binding by putative calmodulin-regulated enzymes. This affinity-chromatography procedure has here been modified for use in demonstrating calmodulin-binding sites on the dyneins in an attempt to rule out possible artifacts and improve its effectiveness. Passage of the sample through Sepharose 4B and CAPP-Sepharose 4B column ensures (*a*) the removal of any aggregates or of material interacting nonspecifically with Sepharose 4B and (*b*) removal of most, if not all, endogenous calmodulin, thus enhancing the specificity and effectiveness of the calmodulin-Sepharose 4B affinity step. One likely contaminant to accompany the eluted dyneins is, of course, any other protein(s) that has a calmodulin-binding site. Analysis of 14S dynein obtained using the serial column procedure on SDS polyacrylamide gels revealed the 14S dynein to be composed of two major high molecular weight polypeptides and one minor high molecular weight component, whereas analysis of the 30S dynein revealed one major and one minor high molecular weight component (see Fig. 6). Both the 14S and 30S affinity-column-purified dyneins contained low levels of lower molecular weight components that may represent (*a*) other components of the dyneins, such as those in dynein I of sea urchin sperm flagella (18); (*b*) other ciliary calmodulin-binding proteins; or (*c*) products of limited proteolysis of the dyneins. It should be noted that although tubulin may be present in the 30S dynein fractions recovered from calmodulin-Sepharose 4B chromatography, no such components were observed in similar preparations of 14S dynein. Because 14S dynein has molecular weight of ~600,000 (20) and is composed primarily of two polypeptides of 260,000 and 253,000 daltons (see Fig. 6) or 358,000 and 375,000 daltons (25), the calmodulin-binding site that was demonstrated to be present by the affinity-column procedure (Fig. 4) is very likely to be localized on one or both of these polypeptides.

Studies with Radiolabeled Calmodulin

The development of a simple procedure for preparing endogenously radiolabeled *Tetrahymena* calmodulin of high specific activity provides a useful tool for studies of calmodulin-binding proteins in *Tetrahymena*. Because of the high degree of structural and functional similarity between *Tetrahymena* calmodulin and bovine brain calmodulin (this paper and reference 28), the [^{35}S]*Tetrahymena* calmodulin should also prove useful for studies on other calmodulin-regulated systems. In the present studies, [^{35}S]calmodulin was used to demonstrate the presence of Ca^{++}-dependent binding sites in twice-extracted axonemal pellets. Although such pellets contain a low amount of Ca^{++}-calmodulin-activatable ATPase activity, it cannot be concluded that the binding sites on pellet 2 are on this residual ATPase, as calmodulin binds Ca^{++}-dependently to an affinity column of tubulin-Sepharose 4B (31). In those experiments, Kumagai et al. (31) used porcine brain tubulin with microtubule-associated proteins (MAPs) to prepare their affinity column. Whether calmodulin interacts directly with tubulin or with the MAPs was not determined in their studies. As it is likely that a twice-extracted axonemal pellet would contain MAPs, the [^{35}S]calmodulin might interact with these proteins in addition to any remaining dyneins or to other, as yet undefined, residual ATPases (3). Further studies will be required to clarify the nature and function of the calmodulin-binding components in the twice-extracted axonemal preparations.

Final Remarks

The in vitro consequences of the Ca^{++}-calmodulin-dependent increase in dynein ATPase activity may be varied. Ca^{++}-dependent changes in symmetry of beat form (2, 15), spontaneous starting and stopping (19, 41), and reversal of beat direction (26, 27) have been observed in demembranated axonemes of different species. We have not found any convincing evidence that addition of calmodulin to demembranated *Tetrahymena* axonemes causes any change in the turbidity response (measured at 350 nm in the presence or absence of added Ca^{++}) even though the addition of Ca^{++} alone consistently causes a small increase in this response. Determination of the means by which the effects of Ca^{++} are relayed by calmodulin (and possibly other dynein-associated regulatory components) to *Tetrahymena* ciliary dynein ATPases and of how this translates into the control of directional ciliary movement awaits further study.

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Preliminary reports of part of these studies have appeared (11, 12, 29).

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