Calcium Control of Waveform in Isolated Flagellar Axonemes of *Chlamydomonas*

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ABSTRACT The effect of Ca^{++} on the waveform of reactivated, isolated axonemes of *Chlamydomonas* flagella was investigated. Flagella were detached and isolated by the dibucaine procedure and demembranated by treatment with the detergent Nonidet; the resulting axonemes lack the flagellar membrane and basal bodies. In Ca^{++}-buffered reactivation solutions containing 10^{-6} M or less free Ca^{++}, the axonemes beat with a highly asymmetrical, predominantly planar waveform that closely resembled that of *in situ* flagella of forward swimming cells. In solutions containing 10^{-4} M Ca^{++}, the axonemes beat with a symmetrical waveform that was very similar to that of *in situ* flagella during backward swimming. In 10^{-5} M Ca^{++}, the axonemes were predominantly quiescent, a state that appears to be closely associated with changes in axonemal waveform or direction of beat in many organisms.

Experiments in which the concentrations of free Ca^{++} and the $CaATP^{--}$ complex were independently varied suggested that free Ca^{++}, not $CaATP^{--}$, was responsible for the observed changes. Analysis of the flagellar ATPases associated with the isolated axonemes and the Nonidet-soluble membrane-matrix fraction obtained during preparation of the axonemes showed that the axonemes lacked the 3.0S Ca^{++}-activated ATPase, almost all of which was recovered in the membrane-matrix fraction.

These results indicate that free Ca^{++} binds directly to an axonemal component to alter flagellar waveform, and that neither the 3.0S CaATPase nor the basal bodies are directly involved in this change.

Calcium ions play a critical role in the control of ciliary and flagellar activity during behavioral responses of a number of organisms. Changes in the internal concentration of Ca^{++} appear to be responsible for ciliary reversal during the avoiding response of *Paramecium* and other ciliates (10, 11), for reversal of the direction of flagellar wave propagation during the avoiding response of the trypanosome *Crithidia* (19, 20), for changes in flagellar waveform during the phototactic response of the green alga *Chlamydomonas* (37), and for arrest of gill cilia in the mussels *Elliptio* (36, 43) and *Mytilus* (28, 41). Calcium also appears to be involved in the flagellar response during chemotaxis of spermatozoids of the bracken fern *Pteridium* (5), and for maintenance of flagellar quiescence in sea urchin sperm (13, 15).

Considerable information is now available on how internal Ca^{++} levels are regulated to bring about changes in ciliary and flagellar movement. Cell physiological, electrophysiological, and genetic studies (see 9, 10, and 23 for reviews) of *Paramecium* have indicated that appropriate stimulation of the cell leads to depolarization of the cell membrane, which in turn results in the opening of voltage-sensitive Ca^{++} channels in the ciliary membrane. Ca^{++} then flows in from the external medium, leading to a high internal concentration of Ca^{++}, and alteration of the ciliary beat. A high internal concentration of Ca^{++} also inactivates the Ca^{++} channels; the internal concentration of Ca^{++} is then rapidly lowered to its resting level by the action of Ca^{++} pumps in the cell membrane, and the cilia return to their normal beat pattern. A similar sequence of events appears to follow photostimulation in *Chlamydomonas* (37).

In contrast, relatively little is known about how or where Ca^{++} acts on the machinery of cilia and flagella to bring about the changes in beat pattern. Several studies have shown that changes in Ca^{++} concentration alter beat pattern in demembranated models (6, 11, 20, 21, 29, 30), indicating that the membrane itself is not directly involved in the changes in waveform. One study has shown that concentrations of Ca^{++} which cause arrest of mussel gill cilia do not inhibit interdoublet

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sliding, which is the basis for ciliary movement (44). However, the mechanical and molecular basis for the Ca^{++} response is still unknown.

To learn more about how changes in internal Ca^{++} levels alter flagellar beat pattern, we have studied the behavior of isolated axonemes of *Chlamydomonas* in reactivation solutions containing different concentrations of Ca^{++}. *Chlamydomonas* is an excellent system for such studies. The Ca^{++} response of the *in situ* flagella has been well characterized and appears to have physiological significance in phototaxis (37); a related response to Ca^{++} has been shown to occur in the isolated flagellar apparatus, which consists of the two flagella together with their basal bodies and associated structures (21). Because axonemes of *Chlamydomonas* can be easily isolated and will reactivate with a waveform closely resembling that of *in situ* flagella (1, 47, 48), it is possible to compare the Ca^{++} response of isolated axonemes with that of intact cells to determine which structural and biochemical components of the flagellar apparatus are involved in the Ca^{++} response.

The results presented here indicate that free Ca^{++} binds directly to an axonemal component to alter flagellar waveform; the Ca^{++} response does not depend upon the basal bodies and their associated structures, or upon the 3.0S Ca^{++}-activated ATPase, which is the major CaATPase in *Chlamydomonas* flagella (46). A possible mechanism for the action of Ca^{++} is discussed.

MATERIALS AND METHODS

Culture Conditions

Chlamydomonas reinhardii strain 1132D$^-$ was synchronously grown in liquid culture as previously described (48). For reactivation experiments, cells were grown in 2-liter Erlenmeyer flasks containing 1.5 liters of Sager and Granick's Medium I (33) modified by addition of three times the normal amount of potassium phosphate; for analyses of flagellar ATPases, cells were grown in 5-liter diphtheria toxin flasks containing 4 liters of the same medium.

Isolation and Reactivation of Axonemes

Cells were harvested and washed as described by Witman et al. (48). Flagella were isolated by the dibucaine procedure (48) and demembranated by resuspension in 1% Nonidet P-40 (BDH Chemicals, Ltd., Poole, England) in ice-cold HMDEKP (30 mM HEPES, pH 7.4 at 22°C; 5 mM $MgSO_4$; 1 mM dithiothreitol[DTT]; 0.5 mM Na_2EDTA; 25 mM KCl; 0.5% polyethylene glycol, 20,000 mol wt). All subsequent operations were carried out at 4°C unless otherwise stated. After demembranation, axonemes were collected by centrifugation at 31,000 *g* for 20 min. The resulting supernate, containing flagellar membrane proteins as well as any soluble components of the flagellar matrix, was used in the analysis of "membrane-matrix" ATPase activities (see below). The axonemes were washed twice by resuspension in HMDEKP followed by recentrifugation. For studies on reactivated axonemes, washed axonemes were resuspended in HMDEKP; an aliquot of the axonemal suspension was then mixed in a 12-ml polypropylene tube with 50–200 vol of one of the Ca^{++}-buffered reactivation solutions described below. Alternatively, an aliquot of axonemes in HMDEKP was combined with an equal volume of a solution which contained appropriate concentrations of components to yield, after dilution with the axonemal suspension, one of the Ca^{++}-buffered reactivation solutions.

Reactivated axonemes were observed and photographed using dark-field optics and carbon-coated slides at 22°C as previously described (48). For quantitation of the percent of axonemes reactivating at the different Ca^{++} concentrations, fields of axonemes were recorded by cinephotomicrography while focusing through the preparation from the slide to the coverslip. Filming was done at 60 frames/s on Kodak 2475 recording film using a Redlake Locam model 51 16-mm high-speed motion picture camera (Redlake Corp., Photo Instrument Div., Campbell, Calif.) synchronized with a Chadwick-Helmuth Strobex power supply and lamp (Chadwick-Helmuth Co., Inc., Monrovia, Calif.). The films were then studied using an L-W model 224-A Photo-Optical Data Analyzer (L-W Photo, Inc., Van Nuys, Calif.) to determine the percent of total axonemes in the field which were beating with symmetrical or asymmetrical waveforms.

Ca^{++}-buffered Reactivation Solutions

The compositions of Ca^{++}-buffered solutions in which the concentration of free Ca^{++} ranged from 10^{-3} to 10^{-9} M were calculated as described by Caldwell (7). Equilibrium constants were obtained from Sillen and Martell (38, 39); the values selected were those measured at 20°C in media containing 0.1 M KCl. Apparent equilibrium constants were calculated for pH 7.3. The compositions of these Ca^{++}-buffered solutions are detailed in Table I. The solutions were designed so that the concentrations of free Mg^{++}, free ATP, and the $MgATP^{--}$ complex remained virtually unchanged from solution to solution and were nearly identical to those in normal reactivation medium (no added Ca^{++}).

In some experiments, the concentrations of free Ca^{++} and the $CaATP^{--}$ complex were manipulated separately to determine the effect of each upon reactivation. Table II shows the compositions of the solutions used in these experiments.

Extraction and Fractionation of ATPases

For determination of the distribution of flagellar ATPases, whole flagella, axonemes, and the membrane-matrix fraction were first dialyzed against TED

TABLE I

Composition of Ca^{++}-buffered Reactivation Solutions *

Final concentrations of components, *mol/liter*								Total concentrations added to solutions, *mol/liter*	
Free Ca^{++}	$CaATP^{--}$	$MgATP^{--}$	Free Mg^{++}	Free ATP	CaEDTA	MgEDTA	Free EDTA	$CaCl_2$	Na_2EDTA
10^{-3}	1.13×10^{-4}	8.52×10^{-4}	3.54×10^{-3}	3.33×10^{-5}	1.76×10^{-2}	6.08×10^{-4}	3.44×10^{-7}	1.87×10^{-2}	1.82×10^{-2}
10^{-4}	1.26×10^{-5}	9.50×10^{-4}	3.54×10^{-3}	3.71×10^{-5}	1.48×10^{-3}	5.10×10^{-4}	2.88×10^{-7}	1.59×10^{-3}	1.99×10^{-3}
10^{-5}	1.27×10^{-6}	9.60×10^{-4}	3.54×10^{-3}	3.75×10^{-5}	1.45×10^{-4}	5.00×10^{-4}	2.82×10^{-7}	1.56×10^{-4}	6.45×10^{-4}
10^{-6}	1.27×10^{-7}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	1.44×10^{-5}	4.97×10^{-4}	2.81×10^{-7}	1.55×10^{-5}	5.12×10^{-4}
10^{-7}	1.27×10^{-8}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	1.44×10^{-6}	4.97×10^{-4}	2.81×10^{-7}	1.55×10^{-6}	4.99×10^{-4}
10^{-8}	1.27×10^{-9}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	1.44×10^{-7}	4.97×10^{-4}	2.81×10^{-7}	1.55×10^{-7}	4.97×10^{-4}
—‡	—	9.63×10^{-4}	3.54×10^{-3}	3.75×10^{-5}	—	5.00×10^{-5}	2.82×10^{-7}	None‡	5.00×10^{-4}
					CaEGTA	MgEGTA	Free EGTA		EGTA
10^{-7}	1.27×10^{-8}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	2.80×10^{-3}	4.97×10^{-4}	1.47×10^{-3}	2.80×10^{-3}	4.77×10^{-3}
10^{-8}	1.27×10^{-9}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	2.80×10^{-4}	4.97×10^{-4}	1.47×10^{-3}	2.80×10^{-4}	2.25×10^{-3}
10^{-9}	1.27×10^{-10}	9.63×10^{-4}	3.54×10^{-3}	3.76×10^{-5}	2.80×10^{-5}	4.97×10^{-4}	1.47×10^{-3}	2.80×10^{-5}	2.00×10^{-3}

* All Ca^{++}-buffered reactivation solutions contained 30 mM HEPES; 5 mM $MgSO_4$; 1 mM DTT; 25 mM KCl; 0.5% polyethylene glycol (20,000 mol wt) and 1 mM total ATP. Solutions were adjusted to pH 7.3 at 22°C after addition of Ca^{++}, ATP, and EGTA.

‡ Standard reactivation solution (48).

TABLE II

Composition of Ca^{++} vs. $CaATP^{--}$ Test Solutions *

Final concentrations of components, *mol/liter*							
Free Ca^{++}	$CaATP^{--}$	Free Mg^{++}	$MgATP^{--}$	MgEDTA	CaEDTA	EDTA	ATP
1×10^{-4}	1×10^{-6}	3.54×10^{-3}	7.56×10^{-5}	5.00×10^{-4}	1.45×10^{-3}	2.82×10^{-7}	2.95×10^{-6}
1×10^{-5}	1×10^{-5}	4.68×10^{-4}	1.00×10^{-3}	5.00×10^{-5}	1.09×10^{-4}	2.13×10^{-7}	2.95×10^{-4}
1×10^{-5}	1×10^{-7}	3.54×10^{-3}	7.56×10^{-5}	5.00×10^{-4}	1.45×10^{-4}	2.82×10^{-7}	2.95×10^{-6}
1×10^{-6}	1×10^{-5}	3.99×10^{-5}	8.53×10^{-4}	5.00×10^{-4}	1.28×10^{-3}	2.50×10^{-5}	2.95×10^{-3}
1×10^{-6}	1×10^{-6}	4.68×10^{-4}	1.00×10^{-3}	5.00×10^{-5}	1.09×10^{-5}	2.13×10^{-7}	2.95×10^{-4}

* All solutions also contained 30 mM HEPES; 1 mM DTT; 25 mM KCl, and 0.5% polyethylene glycol, 20,000 mol wt. Solutions were adjusted to pH 7.3 at 22°C after addition of all components.

(1 mM Tris-HCl, pH 8.3 at 22°C; 0.1 mM EDTA; 0.1 mM DTT) containing 1% Nonidet.[1] Isolated flagella and demembranated axonemes were resuspended in TED-1% Nonidet, whereas the membrane-matrix fraction in HMDEKP-1% Nonidet was put directly into the dialysis tubing. The suspensions were dialyzed overnight at 4°C with one change of dialysis buffer. This procedure solubilized nearly all of the flagellar ATPase activity (46; M. Bessen, unpublished results). Immediately after dialysis, the flagellar and axonemal suspensions were centrifuged at 31,000 *g* for 30 min. Aliquots of each supernate as well as of the dialyzed flagellar membrane-matrix fraction were then layered on separate 5-ml linear sucrose density gradients. Gradients were 5–20% sucrose in 30 mM Tris-HCl (pH 7.5 at 22°C), 0.5 mM EDTA, 1 mM DTT, 25 mM KCl, and 1% Nonidet. The gradients were centrifuged for 4.5 h at 60,000 rpm in an SW-65 Ti-rotor in a Beckman L5-75 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Fractions were collected dropwise from the bottom of the tube and assayed for ATPase activity by a modification of the method of Conway and Lipmann (8 and footnote 2). The final assay mixture contained 30 mM Tris (pH 7.5 at 22°C); 5 mM $MgSO_4$ or 5 mM $CaCl_2$; 1 mM DTT; 0.5 mM EDTA; 25 mM KCl, and 1 mM ATP.

Protein concentrations were determined by the protein-dye binding method of Bradford (4), using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, Calif.) and bovine gamma globulin as a protein standard.

RESULTS

Movement of Reactivated Axonemes in Low Concentrations of Free Ca^{++}

The movements of isolated, reactivated axonemes in the various Ca^{++}-buffered reactivation solutions (Table I) were observed by dark-field light microscopy. In solutions containing 10^{-6} M or less free Ca^{++}, large numbers of axonemes swam in contact with the glass slide or coverslip; these axonemes moved in circles of ~4 μm diameter at speeds of ~2–5 rps (Fig. 1 *A–C*). Approximately 90% of the reactivated axonemes in contact with the coverslip swam in a clockwise direction as viewed from above, whereas ~90% of those in contact with the glass slide circled counterclockwise. There was no change in the tendency of the axonemes to swim in a given direction in different concentrations of free Ca^{++} ranging from 10^{-6} to 10^{-9} M. Axonemes not in contact with the glass slide or coverslip also usually swam in circles of ~4 μm diameter; when such axonemes were swimming in a plane perpendicular to that of the slide, the bends were observed to be confined to the plane of circling. Less frequently, the waves were slightly three-dimensional and caused the axonemes to corkscrew through the medium in a helical path having a short but variable pitch and a diameter of ~4 μm (Fig. 2 *A*); this type of movement was most frequently seen at 10^{-6} M Ca^{++} and observed less frequently at lower concentrations. Corkscrewing was also seen more frequently immediately after preparation of the slide.

The waveform of reactivated axonemes at 10^{-6} M or less free Ca^{++} was highly asymmetrical. Fig. 3 shows tracings of the dark-field images of the right-hand axoneme in Fig. 1 *A–C*; these tracings have been arranged in an order to show the formation of waves at the proximal end of the axoneme and their subsequent propagation to the distal tip.[3] Each wave is composed of a very large principal bend and a barely discernible reverse bend. In such axonemes the angle of the principal bend varied from ~130° to 150°, whereas that of the reverse bend was ~20°–25°.

Reactivated axonemes which were stuck to the slide or coverslip by one end rapidly pivoted around their point of attachment (Fig. 2 *B–D*). The form of the bends in these axonemes was very similar to that of unattached axonemes swimming in contact with the slide or coverslip.

Similar results were obtained regardless of whether the concentration of free Ca^{++} in the reactivation solutions was buffered with EDTA or EGTA (Table I).

Movement of Reactivated Axonemes in High Concentrations of Free Ca^{++}

At 10^{-4} M free Ca^{++}, free swimming axonemes moved rapidly through the medium in straight lines (Fig. 1 *D–F*). Under these conditions, the axonemes propagated nearly symmetrical waves from base to tip. These waves usually appeared to be confined primarily to a single plane, although in some images the waveform was distinctly three-dimensional (Fig. 2*H*). As the axonemes progressed through the medium, the plane of bending rapidly rotated around the axis of propulsion. This was particularly clear using strobed illumination; images in which the axonemes had one or two large, distinct bends rapidly alternated with images in which the axonemes appeared straight, but often were sharply in focus only in their midregion or at their ends (Fig. 1 *D* and *E*). For most axonemes the maximum bend angles were in the range of 80°–90°. Beating axonemes attached to the slide or coverslip by one end either remained in a fixed orientation or pivoted slowly around the point of attachment (Fig. 2 *G*); the waveform of these axonemes was not noticeably different from that of free swimming axonemes in the same solution, except that there were no images in which the axonemes appeared straight.

[1] 1% Nonidet was included in both the dialysis buffer and the sucrose gradients to ensure equivalence between the membrane-matrix fraction, which was already in 1% Nonidet, and the whole flagella and axonemal fractions. The inclusion of 1% Nonidet had no effect on the ability of the low ionic strength dialysis to solubilize the different flagellar ATPases (results not shown).

[2] R. B. Fay and G. B. Witman. Manuscript in preparation.

[3] In high-speed movie films, the proximal end of an isolated axoneme is easily identified as the end at which bends are formed. This end usually appears slightly thicker and brighter than the distal tip, which often tapers to a point. Moreover, the curvature of a bend decreases slightly as it is propagated toward the distal tip. Those latter two characteristics were used to identify the proximal and distal ends of axonemes in still micrographs.

TABLE II, *continued*

Total concentrations added to solutions, *mol/liter*			
$MgSO_4$	$CaCl_2$	Na_2EDTA	ATP
4.12×10^{-3}	1.55×10^{-3}	1.95×10^{-3}	7.96×10^{-5}
1.52×10^{-3}	1.29×10^{-4}	1.59×10^{-4}	1.31×10^{-3}
4.12×10^{-3}	1.55×10^{-4}	6.45×10^{-4}	7.87×10^{-5}
1.39×10^{-3}	1.29×10^{-3}	1.81×10^{-3}	3.82×10^{-3}
1.52×10^{-3}	1.29×10^{-5}	6.11×10^{-5}	1.30×10^{-3}

Fig. 3 shows tracings of the dark-field images of the axoneme in Fig. 2*E–G*; these tracings have been ordered to show the propagation of the waves from base to tip.[3] The bends on opposite sides of the axoneme are nearly equal, in marked contrast to the situation in the presence of 10^{-6} M or less free Ca^{++}.

In 10^{-5} M free Ca^{++}, very little reactivation was observed (see below); however, the majority of those axonemes which did reactivate in 10^{-5} M free Ca^{++} beat with a waveform indistinguishable from that of axonemes in 10^{-4} M Ca^{++}.

In 10^{-3} M free Ca^{++}, a large percentage of the axonemes propagated apparently symmetrical bends, but the beat frequency was very slow (~1 Hz) and no swimming was observed.

Quantitation of Ca^{++} Effect on Axonemal Waveform

The percent of reactivated axonemes having asymmetrical or symmetrical waveforms at different concentrations of free Ca^{++} was determined by directly observing the axonemes in the microscope and scoring the number in several fields which were circling and corkscrewing (asymmetrical waveform) or swimming in straight lines (symmetrical waveform). Fig. 4 shows the results for free Ca^{++} concentrations ranging from 10^{-4} to 10^{-8} M. At concentrations $\leqq 10^{-6}$ M, virtually all of the reactivated axonemes were beating with asymmetrical waveforms. In contrast, at 10^{-5} and 10^{-4} M, at least 90% of the reactivating axonemes were beating with symmetrical waveforms. The shift from asymmetrical to symmetrical waveform occurred abruptly at free Ca^{++} concentrations between 10^{-5} and 10^{-6} M. It should be noted that most of the small percentage of axonemes scored as having an "asymmetrical" waveform at 10^{-5} and 10^{-4} M Ca^{++} were corkscrewing through the medium. The helical paths of these axonemes were of considerably longer pitch than those infrequently observed for free swimming axonemes at 10^{-6} M or less free Ca^{++}; nevertheless, the two types of corkscrewing could not always be distinguished with certainty in direct observations, so all such axonemes were grouped together as having asymmetrical waveforms. However, when axonemes were analyzed by high-speed cinephotomicrography, it was apparent that corkscrewing axonemes at 10^{-5} and 10^{-4} M Ca^{++} had nearly symmetrical but nonplanar waveforms (see below).

Effect of Ca^{++} on Number of Axonemes Reactivated

In the course of the above studies, it was observed that far fewer axonemes reactivated at 10^{-5} M free Ca^{++} than at 10^{-4} or 10^{-6} M or lower. To quantitate the effect of different concentrations of Ca^{++} on the number of axonemes reactivated, the percent reactivation in various Ca^{++}-buffered solutions was determined by analysis of high-speed movie films made while focusing through the preparations from the slide to the coverslip. Fig. 5 shows the percent of total axonemes which beat with asymmetrical or symmetrical waveforms at concentrations of free Ca^{++} ranging from 10^{-4} to 10^{-7} M. The percent of axonemes reactivated decreased from ~50% at 10^{-7} M Ca^{++} to ~20% at 10^{-6} M. At 10^{-5} M, very little reactivation occurred; in some experiments, no reactivation was observed at this concentration of Ca^{++}. At 10^{-4} M, the percent of reactivation increased to over 40%. In these studies, all of the reactivated axonemes had asymmetrical waveforms at 10^{-6} and 10^{-7} M Ca^{++}, whereas all had symmetrical waveforms at 10^{-5} and 10^{-4} M. These results are in good agreement with those obtained by direct observation of the reactivated axonemes.

Effect of Free Ca^{++} vs. $CaATP^{--}$

As the concentration of free Ca^{++} was increased in our Ca^{++}-buffered reactivation solutions, there was a proportionate increase in the concentration of the $CaATP^{--}$ complex (Table I). It was therefore unclear whether a change in the concentration of free Ca^{++} or of $CaATP^{--}$ was responsible for the observed change in axonemal waveform. To determine the independent effect of these two components on axonemal movement, axonemes were reactivated in Ca^{++}-buffered solutions in which free Ca^{++} was varied from 10^{-4} to 10^{-6} M and $CaATP^{--}$ was varied separately from 10^{-5} to 10^{-7} M (Table II). As summarized in Table III, at concentrations of free $Ca^{++} \geqq 10^{-5}$ M, the axonemes always beat with symmetrical waveforms, whereas at most concentrations of free $Ca^{++} \leqq 10^{-6}$ M, the axonemes swam with asymmetrical waveforms, regardless of the concentration of $CaATP^{--}$. The only exception was at 10^{-6} M free Ca^{++}, 10^{-5} M $CaATP^{--}$, in which the axonemes beat symmetrically.

Distribution of Flagellar ATPases

Chlamydomonas flagella contain a number of ATPases, including 12S and 18S dynein-like ATPases which are activated by either Ca^{++} or Mg^{++} (12, 31, 46), and one 3S ATPase which is activated only by Ca^{++} (12, 46). Previous studies have mentioned the 3S Ca^{++}-specific ATPase in the context of Ca^{++} control of flagellar waveform (6, 21, 46). To determine if this enzyme was an axonemal component which might be directly involved in the switch between asymmetrical and symmetrical beating, we compared the relative amounts of this and the other ATPases in whole flagella, in isolated axonemes, and in the membrane-matrix fraction that contained flagellar membrane components plus any soluble components of the flagellar matrix.

When whole flagella of *Chlamydomonas* are dialyzed against low ionic strength buffers, most of the flagellar ATPase activity is solubilized; the ATPases can then be separated by sucrose density gradient centrifugation (46). Fig. 6*A* shows the ATPase profile of such a gradient; the 3S Ca^{++}-specific ATPase occurred as a prominent peak near the top of the gradient, well separated from the Ca^{++}- or Mg^{++}-activated 12S and 18S ATPases. The same dialysis procedure effectively solubilized the ATPases from isolated axonemes previously demembranated by treatment with 1% Nonidet. Sucrose density gradient centrifugation of such extracts revealed that the axonemes contained both of the Mg^{++}-activated ATPases, but only 1.6% of the 3S CaATPase activity present in an equivalent number of whole flagella (Fig. 6*B*). In contrast, the membrane-matrix fraction contained none of the Mg^{++}-activated ATPases, but virtually all of the CaATPase activity (Fig. 6*C*). Before dialysis, the axonemes used in these experiments were capable of undergoing the Ca^{++}-induced change in axonemal waveform.

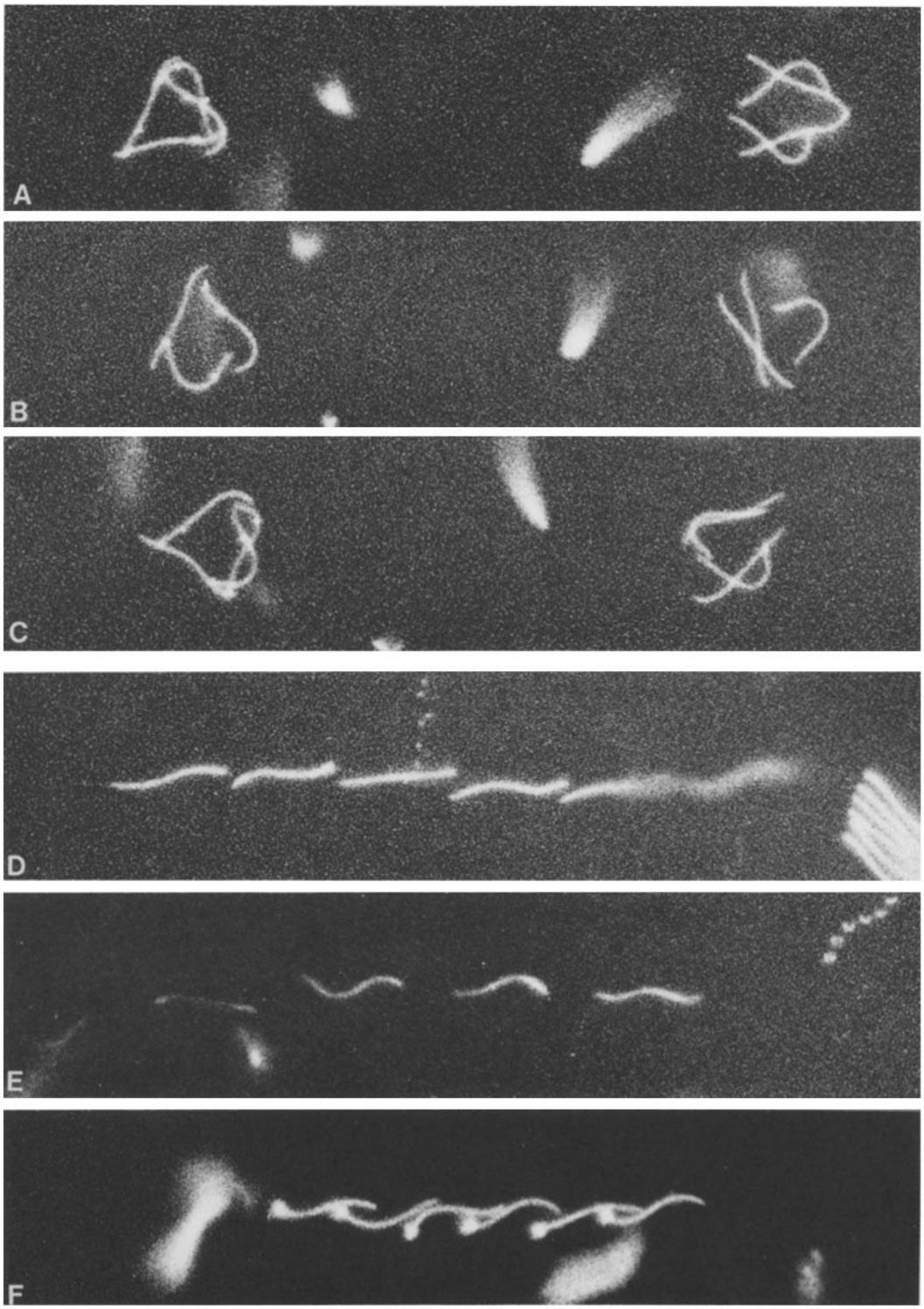

FIGURE 1 (*A–C*) Three multiple-flash dark-field micrographs of two reactivated axonemes circling in contact with the glass slide or coverslip in the presence of 10^{-6} M free Ca^{++}. Flash rate 4 Hz; 1-s exposures. × 4,040. (*D–F*) Multiple-flash micrographs of three different axonemes swimming in straight lines in the presence of 10^{-4} M free Ca^{++}. In some images the axonemes appear nearly straight (third image from left in *D*, fourth image from right in *E*), apparently because bending has occurred in a plane perpendicular to the plane of focus. The axoneme in *F* has a piece of debris or possibly a basal body attached to one end; such axonemes were observed very rarely. Flash rate 6 Hz; 1-s exposures. × 4,040.

DISCUSSION

The experiments reported here demonstrated that isolated axonemes of *Chlamydomonas* undergo two distinctly different types of movement depending on the concentration of Ca^{++} in the reactivation solution. In the presence of 10^{-6} M or less free Ca^{++}, axonemes propagated nearly planar, asymmetrical waves which caused them to swim in circles of relatively small diameter. The waveform under these conditions appeared to be identical to the asymmetrical, ciliary type of beat previously

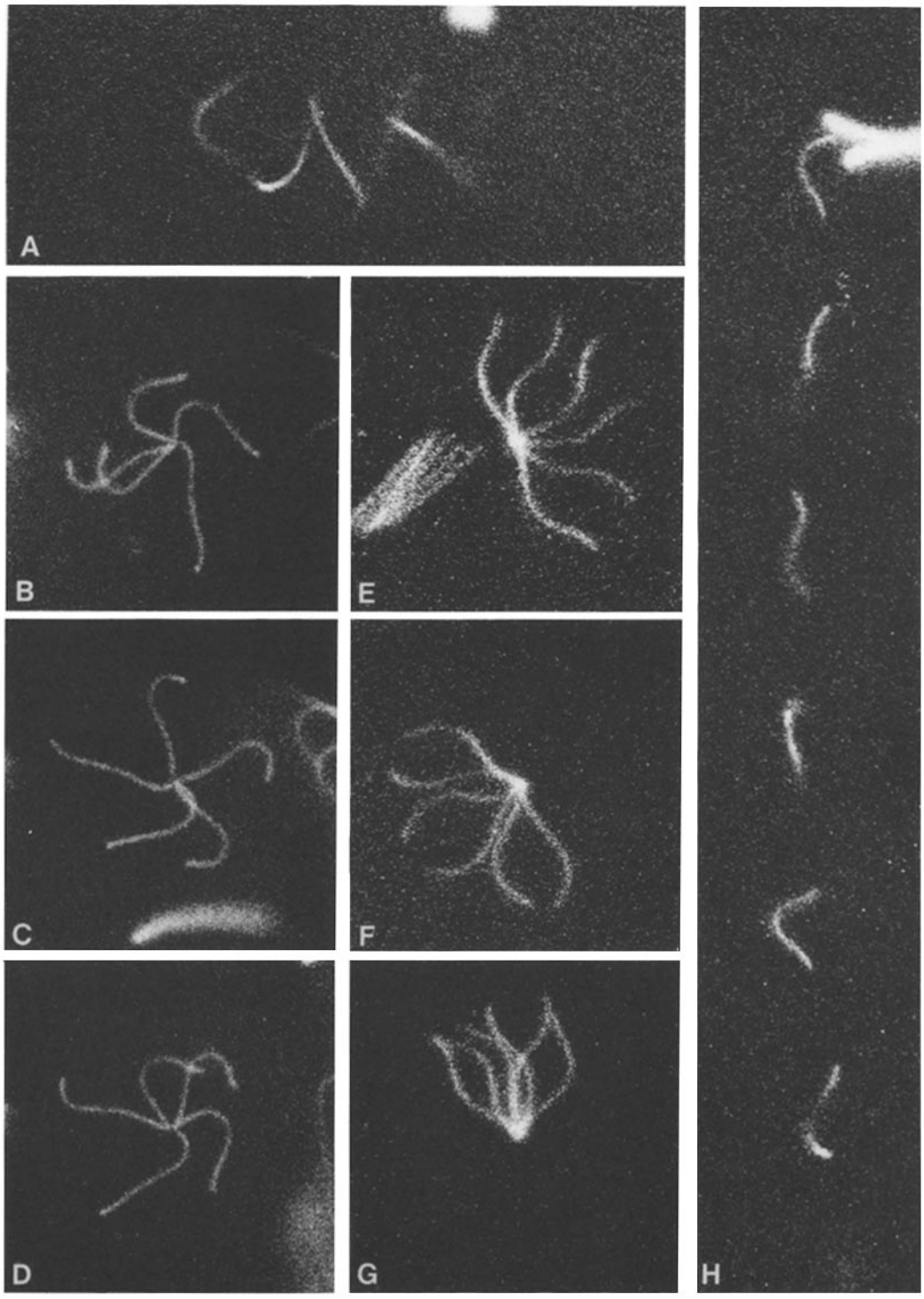

FIGURE 2 Multiple-flash dark-field micrographs of reactivated axonemes. (*A*) An axoneme corkscrewing through the medium in 10^{-6} M Ca^{++}. This axoneme was moving from left to right. Flash rate 5.5 Hz; 1-s exposure. × 4,040. (*B–D*) Three micrographs of an axoneme beating in the presence of 10^{-6} M free Ca^{++}. This axoneme was attached to the coverslip by one end and pivoting rapidly about its point of attachment. Flash rate 10 Hz; ½-s exposures. × 4,040. (*E–G*) Three micrographs of an axoneme beating in the presence of 10^{-4} M free Ca^{++}. This axoneme was attached to the glass slide or coverslip by one end and pivoting slowly about its point of attachment. Note the symmetrical waveform. Flash rate 5 Hz; 1-s exposures. × 4,040 (*H*) A straight swimming axoneme in 10^{-4} M free Ca^{++}. There appears to be a helical component to the waveform. Flash rate 6 Hz; 1-s exposure. × 2,500.

described for *in situ* flagella of forward swimming *Chlamydomonas* (21, 32). In contrast, in the presence of 10^{-4} M free Ca^{++}, axonemes propagated nearly planar symmetrical waves which caused them to move through the medium in straight lines; the waveform under these conditions was very similar to the symmetrical, flagellar-type of beat previously described for *in situ* flagella of backward swimming *Chlamydomonas* (21, 32). At 10^{-5} M Ca^{++}, most of the axonemes were quiescent; occasionally a few axonemes beat symmetrically. Only very slow symmetrical beating was observed at 10^{-3} M Ca^{++}; flagellar move-

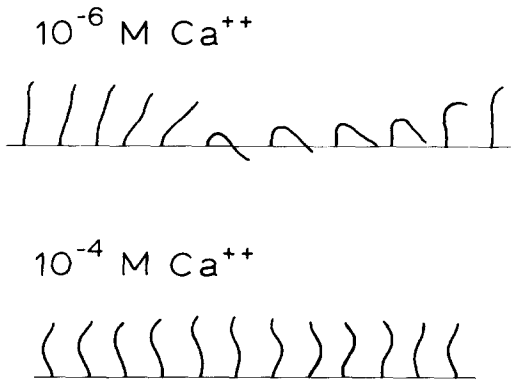

FIGURE 3 Comparison of waveforms of axonemes reactivated in 10^{-6} M free Ca^{++} (top) and 10^{-4} M free Ca^{++} (bottom). Tracings of dark-field images of the right-hand axoneme in Fig. 1 *A–C* (10^{-6} M Ca^{++}) and the axoneme in Fig. 2 *E–G* (10^{-4} M Ca^{++}) have each been arranged in a sequence to illustrate the formation and propagation of bends in the two cases. The proximal ends of the tracings have been placed in contact with the horizontal lines.

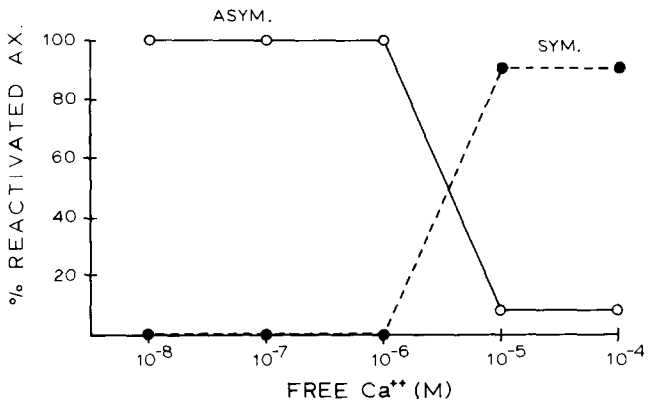

FIGURE 4 The percent of reactivated axonemes beating with asymmetrical (○) or symmetrical (●) waveforms at various concentrations of free Ca^{++}. The number of axonemes scored at each concentration of Ca^{++} varied from 240 to 1,050, except at 10^{-5} M free Ca^{++} where 45 axonemes were scored.

ment is apparently inhibited at such high concentrations of Ca^{++}.

These observations closely parallel the observations of Schmidt and Eckert (37) on the Ca^{++} dependence of the photoresponse of *Chlamydomonas*. Schmidt and Eckert observed that when cells swimming forward in media containing 10^{-3} M Ca^{++} were illuminated with white light, the cells quickly switched to backward swimming. Backward swimming continued for ~200 ms, after which time the cells slowed to a stop, wobbled about their transverse axis for another 200 ms, and then returned to forward swimming. However, when cells swimming forward in 10^{-5}–10^{-6} M Ca^{++} were illuminated, the cells ceased forward swimming, moved their flagella in "interrupted undulations" which resulted in either slow backward swimming or no net cell movement and then, after a few hundred milliseconds, returned to normal forward swimming. Finally, when the same experiment was carried out in the presence of $<10^{-6}$ M Ca^{++}, illumination had no effect on the cells—they continued to swim forward. Similarly, we observed that in the presence of 10^{-4} M Ca^{++}, reactivated axonemes propagated symmetrical waves similar to those associated with backward swimming in whole cells; in 10^{-6} M or less Ca^{++}, they propagated the highly asymmetrical bends associated with forward swimming; and in 10^{-5} M Ca^{++}, they were predominantly quiescent, with only a few axonemes propagating symmetrical waves. The fact that isolated axonemes responded to different concentrations of Ca^{++} in this way is in good agreement with Schmidt and Eckert's conclusion that, after photostimulation, Ca^{++} enters the cell, transiently raising the internal concentration of Ca^{++} and altering the form of the flagellar beat. Our observation that axonemes are quiescent in 10^{-5} M Ca^{++} suggests that the periods of wobbling and interrupted undulatory movement observed by Schmidt and Eckert were probably caused by the internal Ca^{++} concentration being in the 10^{-5}–10^{-6} M range during these times.

Our findings on the Ca^{++} dependence of axonemal waveform are also in basic agreement with the observations of Hyams and Borisy (21) on the effect of Ca^{++} on the waveform of the isolated flagellar apparatus (both flagella plus their basal bodies and associated structures) of *Chlamydomonas*. However, these investigators, using the Ca^{++}-buffered solutions of Brokaw et al. (6), reported that the switch between forward and backward swimming in the demembranated flagellar apparatus occurred between 10^{-6} and 10^{-7} M Ca^{++}. Furthermore, they did not report quiescence of the flagellar apparatus at any intermediate concentration of Ca^{++}, although they did state that in perfusion experiments in which the Ca^{++} concentration was raised or lowered across the threshold for waveform reversal, backward or forward swimming flagellar apparatus ceased to beat before switching to the reversed form of beat. To learn more about the reasons for the differences between our observations and those of Hyams and Borisy, we investigated the behavior of isolated axonemes in the Ca^{++}-buffered solutions of Brokaw (results not shown). Brokaw's solutions differed from our own in a number of parameters, including a higher concentration of KCl (150 vs. 25 mM), lower concentration of free Mg^{++} (0.5 vs. 3.5 mM), and higher pH (8.0 vs. 7.3). In these solutions, the isolated axonemes beat asymmetrically at Ca^{++} concentrations $<10^{-7}$ M, were quiescent at 10^{-6} and 10^{-5} M Ca^{++}, and propagated symmetrical waves at 10^{-4} and 10^{-3} M Ca^{++}, although the beat frequency was very slow at 10^{-3} M. Comparison of these results with those obtained in our own solutions indicate that the response of an isolated axoneme to Ca^{++} is affected by other ions in the medium (see below). The results also suggest that there may be a real difference between isolated axonemes and the isolated flagellar apparatus with regard to

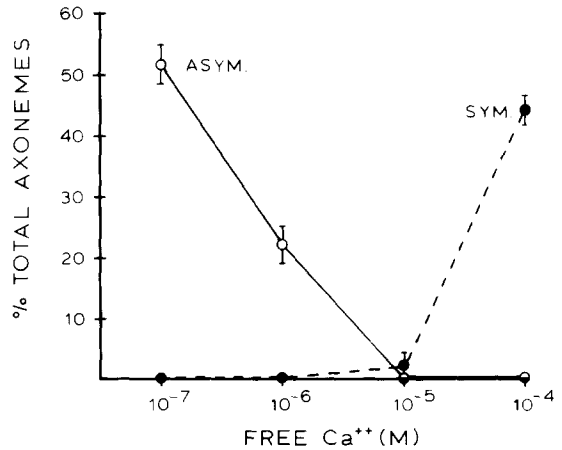

FIGURE 5 The percent of total axonemes which beat with asymmetrical (○) or symmetrical (●) waveforms at the indicated concentrations of free Ca^{++}. Values represent the means of two to five determinations (82–306 axonemes per point) ± SEM.

TABLE III

Predominant Axonemal Waveform at Various Concentrations of Free Ca^{++} and $CaATP^{--}$

	CaATP^{--}, M				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Free Ca^{++}, M					
10^{-3}	S	—	—	—	—
10^{-4}	—	S	S	—	—
10^{-5}	—	S	S	S	—
10^{-6}	—	S	A	A	—
10^{-7}	—	—	—	—	A

S, symmetrical waveform; *A*, asymmetrical waveform.

the range of Ca^{++} concentrations over which quiescence is induced, although confirmation of this would require a direct comparison of the responses of axonemes and flagellar apparatus in the same solutions.

Quiescence such as we observed at 10^{-5} M Ca^{++} appears to be closely associated with changes in the direction or form of the ciliary or flagellar beat. In *Paramecium*, a slight depolarization from the resting membrane potential results in a marked decrease in ciliary beat frequency, often leading to complete ciliary arrest; with further depolarization the frequency of beating greatly increases as the cilia reverse the direction of their effective strokes. Furthermore, cilia recovering from a period of reversed beating induced by a depolarizing stimulus pass through a period of inactivation before returning to normal beating. These changes appear to be linked to changes in the concentration of internal free Ca^{++}, with reduced beat frequency or ciliary arrest occurring at an intermediate Ca^{++} concentration, and increased beat frequency occurring at higher or lower concentrations (24–26). In *Blastocladiella*, the flagella of forward swimming spores propagate symmetrical bends from base to tip. Immediately before turning of the organism, the flagellum becomes quiescent; a large asymmetrical bend is then formed which causes the spore to reorient. The flagellum then returns to a normal symmetrical waveform (27). A similar behavior has been reported for the male gametes of *Allomyces* (20, 27). It would be very interesting to know if quiescence and the change in flagellar waveform in these organisms was dependent upon Ca^{++} concentration in the same way as in *Chlamydomonas* and *Paramecium*.

In contrast to the quiescence induced by intermediate concentrations of Ca^{++} in *Chlamydomonas*, the inhibition of movement observed at 10^{-3} M Ca^{++} may simply reflect the upper limit of physiological tolerance of the motile machinery for Ca^{++}. Cessation of ciliary or flagellar beating in the millimolar Ca^{++} range has been observed in Triton- or glycerol-extracted models of a number of other organisms and cell types (20, 29, 30, 43). The mechanisms of inhibition at intermediate and high Ca^{++} concentrations are not known, nor is it clear if they are related. ATP-induced sliding of trypsin-treated axonemes occurs at both the intermediate (2) and high (42, 44) Ca^{++} concentrations at which arrest occurs, suggesting that these concentrations of Ca^{++} do not induce quiescence by simply inhibiting dynein arm action. However, such studies are not conclusive because the trypsin treatment used to uncouple sliding from bending might also have destroyed components involved in the control of dynein arm activity.

Although the waveform of both asymmetrically and symmetrically beating axonemes appeared to be predominantly planar, the observed patterns of swimming indicated that in both cases there was a slight nonplanar component to the beat. When viewed from above, a large majority of axonemes in 10^{-6} M or less free Ca^{++} circled clockwise when swimming against the coverslip and counterclockwise when against the slide. Similar behavior has been reported for sperm of a number of species (14, 16, 17, 18, and 17 for review of earlier literature). In his analysis of sea urchin sperm movement (17), Sir James Gray provided an explanation for this phenomenon. The movement of a swimming sperm can be discussed in terms of "roll", a rotation about the median longitudinal axis of the

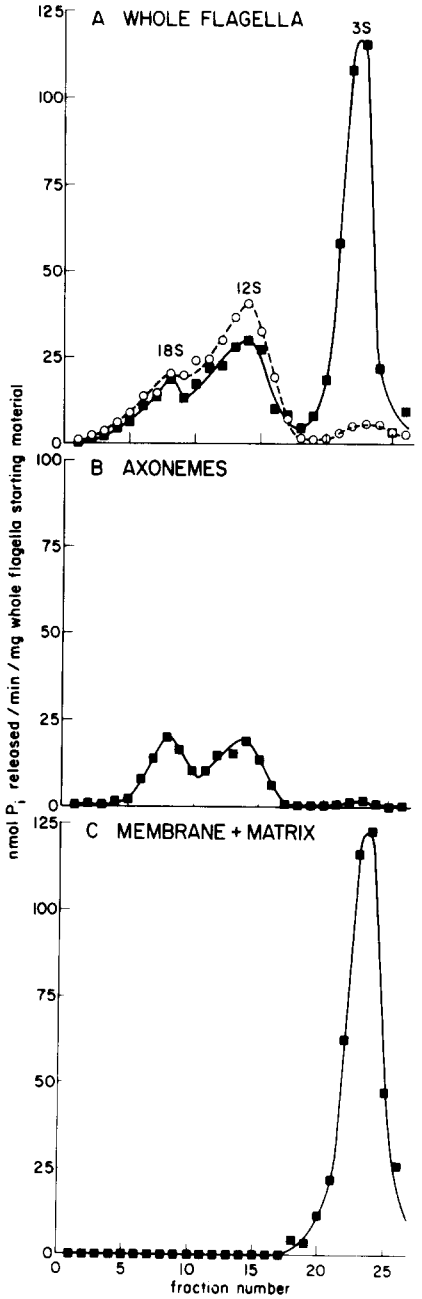

FIGURE 6 Sucrose density gradient fractionation of ATPases in soluble fractions obtained by low ionic strength dialysis of: (*A*) whole flagella; (*B*) isolated axonemes; (*C*) the membrane-matrix fraction. ATPase activity was measured in the presence of 5 mM Ca^{++} (■) or 5 mM Mg^{++} (○) and is plotted as nanomoles P_i released per minute per milligram of whole flagella starting material. The 3S, 12S, and 18S peaks (46) were identified by their positions relative to that of catalase in similar gradients (results not shown).

head; and "yaw", a rotation about an axis running through the head and perpendicular to the plane of beat. A sperm which only yaws will swim in a circular arc; one which only rolls will swim in a straight line; and one which both yaws and rolls will swim in a helix, the handedness of which will depend on the direction of rolling and is independent of the original direction of yawing. If the forward movement of a sperm which is yawing and rolling is blocked by a surface such as a glass slide, the sperm will begin circling against the surface in the same direction as the original direction of roll. Thus, the tendency of sperm to circle counterclockwise when viewed towards the surface over which they are moving is a result of the sperm originally having had a roll which was counterclockwise as viewed from behind. This reasoning is also applicable to reactivated *Chlamydomonas* axonemes; because 80–90% of the axonemes circled counterclockwise against the glass slide, a large majority of the axonemes must originally have had a counterclockwise roll as viewed from a distal to proximal direction. Gray was unable to determine whether the tendency of sperm to roll counterclockwise was due to an asymmetry of the sperm head or to a slight nonplanar component in the sperm's beat. However, in the case of the isolated *Chlamydomonas* axoneme, the movement must be caused by a nonplanar beat. Slightly nonplanar bends were sometimes evident in axonemes swimming freely in the media (Fig. 2*A*); presumably, the majority of axonemes circling in contact with the slide or coverslip originally had a similar waveform. The axonemal bends must have had a right-handed twist to give rise to a counterclockwise roll.

In symmetrically beating axonemes swimming in straight lines through media containing 10^{-5} or 10^{-4} M free Ca^{++}, the plane of bending rapidly rotated about the axis of propulsion. This rotation of the bend plane was probably caused by the axonemes again rolling about their longitudinal axes as a result of a slight nonplanar component in the waveform. Nonplanar bends were observed in some axonemes (Fig. 2*H*); although other axonemes did not have a noticeably helical waveform, Hiramoto and Baba (18) have calculated that rolling of a flagellum with an apparently planar beat could be caused by a nonplanar component too small to detect by light microscopy. It is unlikely that the plane of bending was changing within the axoneme itself, because the plane of bending did not change in axonemes stuck to the coverslip by one end (Fig. 2*E–G*).

To determine whether free Ca^{++} or $CaATP^{--}$ was the component controlling axonemal waveform, we observed the movement of axonemes in reactivation solutions in which the two components were varied separately. The results indicated that free Ca^{++}, not $CaATP^{--}$, was responsible for the change in waveform, with the switch between asymmetrical and symmetrical beating occurring between 10^{-5} and 10^{-6} M free Ca^{++}, regardless of the concentration of $CaATP^{--}$ (Table III). The only exception was in the solution containing 10^{-6} M free Ca^{++} and 10^{-5} M $CaATP^{--}$, in which the axonemes beat symmetrically. This latter response may have been caused by the concentration of free Mg^{++} in this solution, which contained 10- to 100-fold less free Mg^{++} than any of the other solutions used (Table II). Decreased Mg^{++} might well decrease the threshold at which the change between symmetrical and asymmetrical beating occurs, especially if the switch involves displacement of a bound Mg^{++} by a Ca^{++} at some critical site.

Several investigators have suggested that the 3.0S Ca^{++}-activated ATPase of *Chlamydomonas* flagella might be directly involved in the switch between asymmetrical and symmetrical beating. However, we found that isolated axonemes retained only ~1% of the amount of this enzyme present in whole flagella, the remainder being in the Nonidet-soluble membrane-matrix fraction. Since these axonemes were capable of undergoing the Ca^{++}-induced change in axonemal waveform, the 3.0S CaATPase cannot be the axonemal component responsible for the change. Studies now in progress indicate that the 3.0 S CaATPase is associated with the flagellar membrane.[4] It may be a Ca^{++} pump involved in regulating the internal concentration of free Ca^{++}; alternatively, it may be involved in the recently described saltatory movement of particles associated with the flagellar surface of *Chlamydomonas* (3).

The experiments described here show for the first time that the Ca^{++}-induced change in flagellar waveform in *Chlamydomonas* does not require the basal body or its associated structures, because axonemes isolated by the dibucaine method are detached at the level of the transitional region and hence lack the basal bodies (35, 48). However, it should be noted that there are probably other Ca^{++}-induced, motility-related changes which do involve the basal bodies. Hyams and Borisy (21) reported that Ca^{++} reversibly induced a change in angle between the two flagella of the isolated flagellar apparatus of *Chlamydomonas*, even in the absence of flagellar beating. We suspect that these changes were caused by a Ca^{++}-dependent contraction of the striated fibers associated with the basal body, similar to the reversible, Ca^{++}-induced contraction observed in striated fibrous roots attached to the basal bodies of *Platymonas* (34). Similarly, Naitoh and Kaneko (29, 30) have shown that, in the absence of Mg^{++}, addition of Ca^{++} and ATP caused the nonmotile cilia of Triton-extracted models of *Paramecium* to switch from a frontward pointing direction to a rearward pointing orientation. It would be interesting to know whether this change involved an actual alteration of the bend form of the cilia, or if it too was the result of a Ca^{++}-induced contraction of a basal body root structure.

The above results indicate that free Ca^{++} binds directly to an axonemal component to alter the form of the flagellar beat in *Chlamydomonas*. We know nothing about the nature of this component. The Ca^{++}-binding protein calmodulin occurs in axonemes of *Tetrahymena* (22) and *Chlamydomonas*,[5] but it has not yet been determined if it is involved in the control of axonemal waveform. Nor do we know where the Ca^{++}-binding component is located within the axoneme. The Ca^{++}-induced change from asymmetrical to symmetrical bend formation and propagation must involve a change in the pattern of interdoublet sliding. Ca^{++} thus might act on the dynein arms, which generate this sliding (40), or on the nexin links or radial spokes and central tubules, which have been implicated in bend formation and the control of sliding (40, 45, 48). A likely possibility for the site of action of Ca^{++} is the central tubule-central sheath complex. In *Chlamydomonas*, one central tubule has two rows of long projections and the other has two rows of short projections (48). Because radial spoke-central sheath interactions are involved in axonemal bend formation (45, 48), this central sheath asymmetry could result in different radial spoke-central sheath interactions in opposite halves of the axoneme, leading to the asymmetrical bends observed at low concentrations of Ca^{++}. In high concentrations of Ca^{++}, Ca^{++}

[4] K. Pfister and G. Witman. Unpublished results.

[5] S. E. Gitelman and G. B. Witman. 1980. Purification of calmodulin from *Chlamydomonas*. Calmodulin occurs in cell bodies and flagella. Manuscript in preparation.

might bind to the central sheath, altering it in such a way that the radial spoke-central sheath interactions become equivalent in the two halves of the axoneme, leading to symmetrical bending. Alternatively, Ca^{++} might bind to one of the other components involved in the generation or control of sliding, releasing these components from the effect of the asymmetrical interactions between the radial spokes and central sheath. We are presently investigating these possibilities by studying mutants of *Chlamydomonas* which are defective in the Ca^{++} control of axonemal waveform.

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