Modulation of the Asymmetry of Sea Urchin Sperm Flagellar Bending by Calmodulin

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ABSTRACT Sea urchin spermatozoa demembranated with Triton X-100 in the presence of EGTA, termed potentially asymmetric, generate asymmetric bending waves in reactivation solutions containing EGTA. After they are converted to the potentially symmetric condition by extraction with Triton and millimolar Ca^{++}, they generate symmetric bending waves in reactivation solutions containing EGTA. In the presence of EGTA, their asymmetry can be restored by addition of brain calmodulin or the concentrated supernatant obtained from extraction with Triton and millimolar Ca^{++}. These extracts contain calmodulin, as assayed by gel electrophoresis, radioimmunassay, activation of brain phosphodiesterase, and Ca^{++}-dependent binding of asymmetry-restoring activity to a trifluorophenothiazine-affinity resin. Conversion to the potentially symmetric condition can also be achieved with trifluoperazine substituted for Triton during the exposure to millimolar Ca^{++}, which suggests that the calmodulin-binding activity of Triton is important for this conversion.

These observations suggest that the conversion to the potentially symmetric condition is the result of removal of some of the axonemal calmodulin and provide additional evidence for axonemal calmodulin as a mediator of the effect of Ca^{++} on the asymmetry of flagellar bending.

Extensive evidence that ciliary and flagellar bending is regulated by intracellular calcium concentrations has been obtained, especially from experiments that use demembranated or membrane-permeabilized cilia or flagella to demonstrate the modulation of motility by changing the calcium concentration of the reactivation medium. A role for calmodulin in these responses to calcium has been suggested, principally based on evidence for the presence of calmodulin or calmodulin-like proteins in cilia and flagella (18, 20, 26, 31, 33, 41, 44). Experiments with calmodulin-binding drugs such as trifluoperazine (TFP)[1] have provided supporting evidence in some systems (37, 38, 49), but in other systems, these agents appeared to have little or no effect at the micromolar concentrations at which they bind to calmodulin (41). In addition, Verdugo et al. (45) reported an increased effect of calcium on the beat frequency of demembranated cilia of tracheal cells by adding exogenous calmodulin.

With sea urchin sperm flagella, the situation is complicated by the observation that the response of demembranated flagella to calcium depends on the previous exposure of the flagella to calcium (7). Sea urchin spermatozoa demembranated with Triton X-100 in the presence of EGTA have "potentially asymmetric" flagella (15). They swim in circular paths with asymmetric flagellar bending patterns when they are diluted into low-Ca^{++} reactivation solutions containing EGTA and $MgATP^{--}$ (3, 7). In reactivation solutions with higher Ca^{++} concentrations, they become even more asymmetric, and in some cases, quiescent (7, 15).

Sea urchin spermatozoa demembranated with Triton X-100 in the presence of millimolar Ca^{++} concentrations have "potentially symmetric" flagella (7). These spermatozoa swim with much straighter paths and nearly symmetric flagellar bending waves when reactivated at low Ca^{++} concentration, and under these conditions their motility more closely resembles that of intact spermatozoa (3, 7). They also show increasing asymmetry as the Ca^{++} concentration of the reactivation solution is increased (3, 7).

Potentially asymmetric sperm flagella can be converted to the potentially symmetric condition by addition of millimolar Ca^{++} at any time after demembranation. The rate of this conversion is greatly reduced when the Triton concentration or the temperature is reduced (35).

Potentially symmetric sperm flagella can be reconverted back to the potentially asymmetric condition by addition of EGTA to lower the Ca^{++} concentration, but only if this is done before substantial dilution of the Triton-demembranated spermatozoa (35). This observation suggested that the conversion of the sperm flagella from potentially asymmetric

[1] *Abbreviation used in this paper:* TFP, trifluoperazine.

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to potentially symmetric by exposure to millimolar Ca^{++} and Triton is associated with removal of a component from the flagella that could rebind and restore asymmetry at low Ca^{++} concentration if a sufficiently high concentration of the component was maintained (35). The present work began as an effort to identify such a component in Triton-Ca^{++} extracts of flagella. Several recent studies have provided evidence that the supernatant fraction solubilized by Triton X-100 or Nonidet P40 contains components that are required for maximal activation of flagellar motility by cAMP-dependent phosphorylation (25, 36, 42). The presence of calmodulin in the detergent-solubilized fraction has also been demonstrated (41). The present paper presents evidence indicating that the removal of calmodulin from the axoneme by detergent extraction in the presence of millimolar Ca^{++} induces the potentially symmetric state and that the potentially asymmetric state can be restored by exogenous calmodulin as well as by the detergent-solubilized fraction.

MATERIALS AND METHODS

Reactivation of Sperm Flagellar Motility: New methods have been developed for reactivation of motility of spermatozoa from the sea urchin *Lytechinus pictus* to obtain high-quality motility in reactivation solutions that contain 1 mM ATP, with acetate as the major anion instead of chloride (4). This anion substitution has previously been shown to give increased bend angles and beat frequencies (17), but it also increases the requirement for activation by cAMP because the activating effect of chloride (4, 36) is eliminated.

Concentrated spermatozoa, stored at 0°C, are demembranated by diluting 1 μl spermatozoa with 100 μl demembranation solution that contains 0.25 M KCl, 2 mM $MgSO_4$, 1 mM EGTA, 1 mM dithiothreitol, 0.04% Triton X-100, and 10 mM Tris-HCl buffer, at pH 8.2. All work is carried out at 18°C. After 30 s, this mixture is diluted with 300 μl of activation solution that contains 0.05 M KCl, 0.5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM ATP, 0.01 mM cAMP, and 10 mM Tris-HCl buffer at pH 8.2. The spermatozoa are incubated in this solution for 3 min. This procedure produces potentially asymmetric spermatozoa, with essentially 100% motility, when they are examined after a 1:100 dilution with reactivation solution that contains 0.25 M K acetate, 3 mM $MgSO_4$, 1 mM EGTA, 1 mM dithiothreitol, 0.5% polyethylene glycol, and 20 mM Tris-HCl buffer at pH 8.2. If cAMP and ATP are omitted from the activation solution, 50–90% of the spermatozoa are quiescent when observed in reactivation solution (4).

Potentially symmetric spermatozoa are produced by the addition of $CaCl_2$ at the end of the incubation with cAMP to give a concentration of 2.5 mM, and an incubation for 20 or 30 s more before dilution into reactivation solution.

Analysis of Flagellar Bending: Reactivated spermatozoa that were swimming at the upper surface of an open drop on a microscope slide were photographed on moving film at a magnification of 64 with strobe flashes at 150 Hz. The negatives were projected onto the screen of a microfilm reader, and four to six images, which covered one beat cycle, were digitized manually to enter the images into a Hewlett-Packard 9816 microcomputer (Hewlett-Packard Co., Palo Alto, CA). The bending patterns for one beat cycle were fitted by model bending patterns generated by a modified constant curvature model. This parameter fitting was done by using the Simplex algorithm to vary the model parameters and to find parameters that minimize the root mean square differences between the curvatures of the flagellar images and the model. This process provides a set of parameters that describes each bending pattern. We were particularly interested in the asymmetry, $\Delta\theta$, which was taken to be the difference between the magnitudes of the principal and reverse bend angles (3). We routinely photographed 25–30 spermatozoa in each experimental preparation and analyzed the first 20 spermatozoa in each experiment that had usable image sequences. Full details of these procedures are published elsewhere (5).

These procedures may overestimate the values of asymmetry for potentially symmetric spermatozoa. First, spermatozoa that swim in straight paths may be underrepresented in the samples because they are difficult to photograph and may swim to the edges of the drop and be trapped there. Second, in experiments in which the asymmetry of flagellar bending patterns was reduced by trypsin (8), cases in which $\Delta\theta$ was reduced continuously from a positive value through zero to a negative value were observed. If this occurred in any of the procedures used here, it would not have been detected, and all values of $\Delta\theta$ were assumed to be positive.

Preparation of Calmodulin Extracts: For quantitative preparation of sperm extracts, spermatozoa were first diluted with cold 0.5 M NaCl until a 10-μl aliquot suspended in 5.0 ml of 0.5 M NaCl gave an optical density (OD) reading of 0.20 at 540 nm in a 12-mm cuvette (8). This suspension was then diluted 1:50 with demembranation solution, followed after 30 s by a 1:3 dilution with activation solution. Subsequent procedures were done at 0–4°C. The suspension was centrifuged for 5 min at 45,000 *g*. The supernatant, S1, was removed and the pellet was resuspended in the same volumes of demembranation and activation solutions used originally. $CaCl_2$ was then added to a concentration of 2.5 mM. The suspension was centrifuged again for 10 min, and the supernatant, S3, was removed.

Modified versions of this procedure were used to prepare extracts from spermatozoa and from isolated flagella of *L. pictus* or another sea urchin, *Strongylocentrotus purpuratus*. Flagella were isolated by the method of Brokaw and Benedict (6). For some of these extractions, larger quantities of spermatozoa or flagella were extracted at lower dilutions. In some preparations, the first sperm pellet was resuspended and washed with demembranation and activation solutions without $CaCl_2$ to produce supernatant S2 before extraction with Ca^{++}. In most cases, 0.5 mM *N* α-*p*-tosyl-L-arginine methyl ester (TAME) (T4626, Sigma Chemical Co., St. Louis, MO) was included in these solutions to retard proteolysis, but no evidence was obtained that it was effective.

Extracts were also obtained by heat treatment of whole spermatozoa, flagella, or partially extracted pellets. For these extractions, each sample was suspended in 2.0 ml of borate-EGTA buffer that contained 125 mM $Na_2B_4O_7$, 5 mM EGTA, and 0.075 mM NaCl at pH 8.4 (10) in a 25-ml polycarbonate centrifuge tube. All samples in an experiment were simultaneously placed in a boiling water bath for 10 min, then cooled on ice. All of these experiments included a tube that contained a known amount of brain calmodulin in 2.0 ml borate-EGTA buffer containing 2 mg/ml bovine serum albumin (BSA). After they were heated and cooled, the samples were centrifuged for 15 min at 45,000 *g*, and the supernatants were decanted. These supernatants were used for radioimmunoassays without further concentration.

For functional calmodulin assays, extracts were concentrated by ultrafiltration by using Amicon PM 10 membranes at 50 psi and Amicon centrifugal microconcentrators (Amicon Corp., Danvers, MA) to reduce the volume to 50–100 μl. S1 and S3 extracts were centrifuged to remove precipitated material after the initial ultrafiltration to ~2 ml.

Radioimmunoassay for Calmodulin: Radioimmunoassays (10) were done by using materials and procedures of the calmodulin radioimmunoassay kit from New England Nuclear (Boston, MA). The standard curve was obtained by using the brain calmodulin samples boiled along with the unknown samples at four concentrations that covered a 10-fold range of concentrations. The unknown samples were assayed at two or three concentrations selected to fall within the range of the standard curve. Two or three tubes were assayed at each concentration.

Phosphodiesterase Activation Assay for Calmodulin: Phosphodiesterase activation was measured as described by Wallace et al. (46) except that the hydrolyzed AMP was measured by a colorimetric assay for inorganic phosphate using malachite green (9). Bovine brain phosphodiesterase was mixed with phosphodiesterase assay buffer (46) containing 50 μM $CaCl_2$ immediately before use, and 0.29 ml of this mixture was placed in each assay tube. Calmodulin standard solutions or samples of extracts, 1–10 μl, were added to each tube, and the tubes were placed in a 30°C water bath. The reaction was initiated by addition of 10 μl 0.05 M cAMP and terminated by the transfer of each tube to a 100°C water bath for 1 min. 10 μl of a 1 mg/ml solution of *Crotalus atratus* venom (P4506, Sigma Chemical Co.) was added to each tube to hydrolyze the AMP formed by the phosphodiesterase. After 10 min, 0.7 ml of the HCl-molybdate reagent and 0.3 ml of the malachite green-polyvinylacetate reagent (9) were added, followed 2 min later by 2.0 ml of 7.8% H_2SO_4 reagent (9). The OD was read 40–60 min later at 625 nm. Under these conditions, 25 nm of AMP gave an OD value of ~0.65.

ATP, used in the preparation of the sperm extracts, will interfere with this assay. We avoided this problem either by running blank tubes that contained extract samples, without phosphodiesterase, and correcting the phosphodiesterase results, or, preferably, by washing the extracts on the microconcentrator membranes with 2 ml of phosphodiesterase assay buffer.

In each assay, a standard curve was constructed by using five concentrations of brain calmodulin. A standard model for saturation kinetics (similar to Eq. 1), which uses three parameters (nonactivated activity, maximum activated activity, and calmodulin concentration for 50% activation), was fitted to this data by using a nonlinear least squares fitting procedure. This curve was then used to obtain equivalent calmodulin concentrations for the levels of phosphodiesterase activity measured with each of the unknown samples. Each unknown sample was assayed at two or three concentrations, and the resulting equivalent calmodulin concentrations were averaged.

Miscellaneous Methods: We determined protein concentrations with the Coomassie Blue dye-binding method by using the Bio-Rad reagent

(Bio-Rad Laboratories, Inc., Richmond, CA) and BSA as a routine standard. Results were multiplied by a factor of 2.0 so that they would be comparable to results obtained in earlier work using the Lowry method with BSA standards.

SDS PAGE was done with standard methods (21, 29) by using 15% polyacrylamide gels. Gel lanes were spaced at 12-mm intervals, and the gel thickness was 1 mm. The silver staining method of Morrissey (32) was used at low protein loadings.

Reagents: Bovine brain calmodulin (P2277) and bovine brain phosphodiesterase (P9529) were purchased from Sigma Chemical Co. TFP was a gift of Smith-Kline Laboratories (Philadelphia, PA).

RESULTS

Preliminary Observations

To obtain an enriched preparation of the putative component responsible for the potentially asymmetric condition, spermatozoa of *L. pictus* were demembranated and activated with cAMP, collected by centrifugation, rinsed, and resuspended in the same mixture of fresh demembranation and activation solutions. $CaCl_2$ (2.5 mM) was then added, and the suspension was recentrifuged. The supernatant (S3) was concentrated by ultrafiltration.

Typical flagellar bending patterns of reactivated spermatozoa of *L. pictus* are illustrated by the multiple exposure photographs in Fig. 1. Fig. 1 *a* shows a spermatozoon from a standard preparation of potentially asymmetric spermatozoa. The measured value of asymmetry, $\Delta\theta$, for this spermatozoon is 1.7 rad. The mean asymmetry for 20 spermatozoa in this sample is $\Delta\theta = 1.67$ rad ± 0.29 rad (S D). Fig. 1 *b* illustrates the result obtained after addition of 2.5 mM $CaCl_2$ to the demembranated, activated spermatozoa and incubation for a further 20 s to produce potentially symmetric spermatozoa. The measured value of $\Delta\theta$ for this spermatozoon is 0.4 rad, and the mean $\Delta\theta$ for 20 spermatozoa in this sample is 0.44 ± 0.29 rad.

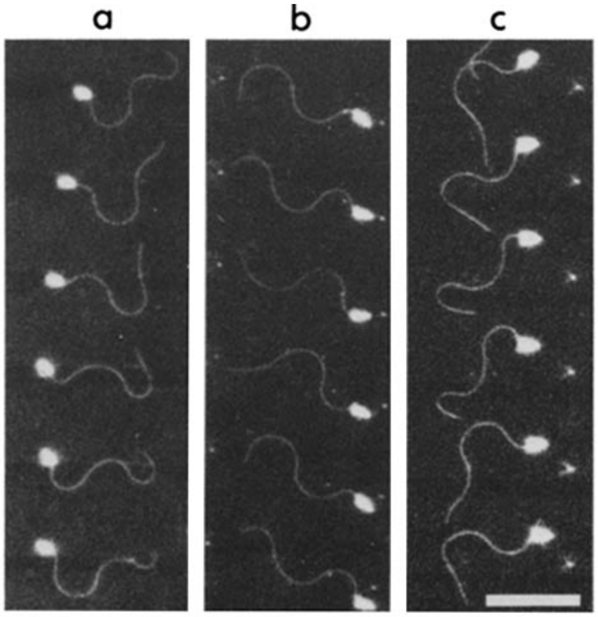

FIGURE 1 Triton-demembranated sea urchin spermatozoa (*Lytechinus pictus*) swimming at the upper surface of an open drop of ATP-reactivation solution. Low resolution photographs for analysis of flagellar bending wave parameters were taken on film moving at 0.25 m/s with strobe flashes at 150 Hz. The scale bar in c indicates 25 μm. (*a*) Standard preparation of potentially asymmetric spermatozoa. (*b*) Spermatozoa prepared as in *a* and then extracted for 20 s by addition of 2.5 mM $CaCl_2$. (*c*) Spermatozoa prepared as in *b* and examined in reactivation solution containing concentrated Ca-Triton extract (0.02 ml/ml).

Fig. 1 *c* illustrates the result obtained by the preparation of potentially symmetric spermatozoa, as in Fig. 1 *b*, and reactivation in a solution containing some of the concentrated supernatant (S3) prepared as described above. In this case, the concentrated supernatant was diluted 1:50 with reactivation solution. The 1 mM EGTA in the reactivation solution is easily adequate to maintain a low Ca^{++} concentration ($<10^{-9}$ M) in spite of the addition of 0.05 mM Ca^{++} with the supernatant. The spermatozoon shown in Fig. 1 *c* has a measured $\Delta\theta$ of 1.5 rad, and the mean $\Delta\theta$ for 20 spermatozoa in this sample is 1.49 ± 0.37 rad. Most of the original asymmetry of these spermatozoa has been restored by exposure to a small amount of the concentrated supernatant.

Several observations indicated that the active component in the S3 supernatant was a calmodulin-like protein. The activity was found to be heat stable and trypsin sensitive. Analysis of the supernatant by SDS PAGE indicated that it was enriched in a component that had calcium-dependent mobility values similar to those of bovine brain calmodulin (Fig. 2). This component is not readily detectable in gels of whole spermatozoa or axonemes (data not shown), in agreement with previous studies of flagellar calmodulin (18, 41). The asymmetry-restoring activity of the supernatant was removed by exposure to a trifluorophenothiazine-Sepharose affinity resin (Caabco, Inc., Houston, TX) in the presence of calcium and was recoverable by addition of EGTA. In one of these binding experiments, we used a sperm preparation that gave $\Delta\theta = 1.91 \pm 0.22$ rad before exposure to 2 mM Ca^{++} and $\Delta\theta = 0.72 \pm 0.34$ rad for the potentially symmetric spermatozoa obtained after exposure to millimolar Ca^{++}. Addition of a concentrated S3 supernatant to reactivation solution (1 part in 300) caused the asymmetry of these potentially symmetric spermatozoa to increase to $\Delta\theta = 1.25 \pm 0.44$ rad. After 100 μl of concentrated supernatant was incubated with 50 μl of the trifluorophenothiazine-Sepharose resin in the presence of 1 mM $CaCl_2$, the supernatant was decanted and added to reactivation solution at a dilution of 1 part in 200. Potentially asymmetric spermatozoa added to this reactivation solution showed no increase in asymmetry ($\Delta\theta = 0.72 \pm 0.33$ rad). The resin was then incubated with 100 μl of solution containing 4 mM EGTA, and the supernatant was decanted and added to reactivation solution at a dilution of 1 part in 200. Potentially asymmetric spermatozoa added to this reactivation solution showed an increase in asymmetry ($\Delta\theta = 1.23 \pm 0.42$ rad) comparable to that obtained with the original supernatant. This behavior is fully consistent with the hypothesis that the active asymmetry-restoring component of the extract is calmodulin, but it is not definitive evidence because of the known lack of specificity of trifluorophenothiazine binding (11, 39). Additional evidence for identification of the asymmetry-restoring component as calmodulin was obtained from reactivity with anti-calmodulin antibody and from activation of phosphodiesterase activity, as described in a later section.

Responses of Demembranated Spermatozoa to Brain Calmodulin

Bovine brain calmodulin is also completely effective in restoring asymmetry to potentially symmetric spermatozoa. A typical dose-response curve is shown in Fig. 3*a*. A near-maximal response is consistently obtained with a calmodulin

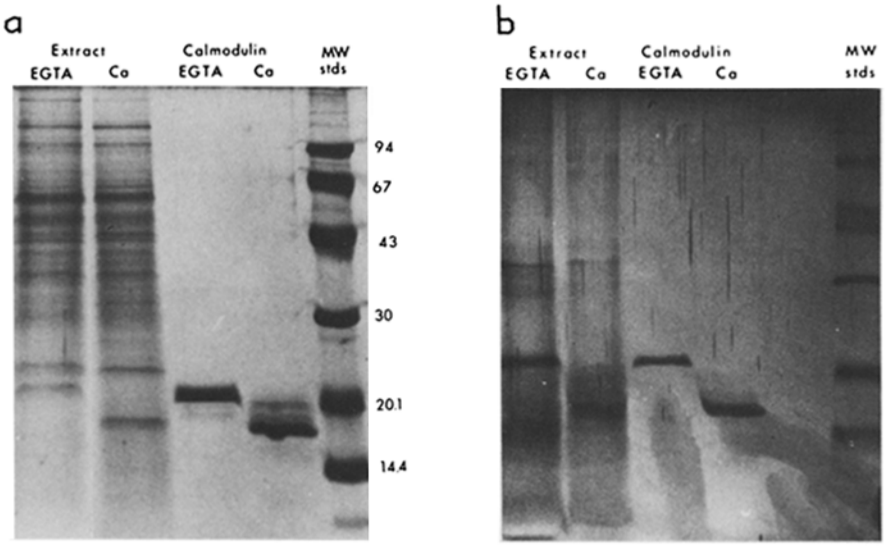

FIGURE 2 SDS PAGE of Triton-calcium extracts (S3) prepared from *S. purpuratus* spermatozoa. (*a*) Extracts prepared from whole spermatozoa (70 μg protein/lane) compared with bovine brain calmodulin (12 μg protein/lane); Coomassie Blue staining. Samples were incubated with 1 mM $CaCl_2$ or 4 mM EGTA, as indicated, before preparation for electrophoresis. (*b*) Extracts (S3) prepared from *S. purpuratus* axonemes after two extractions with Triton and EGTA (S1, S2) (0.5 μg protein/lane) compared with bovine brain calmodulin (0.05 μg/lane); silver staining. Samples were incubated with 4 mM $CaCl_2$ or 4 mM EGTA, as indicated, before preparation for electrophoresis. Molecular weights for protein standards are given in kilodaltons.

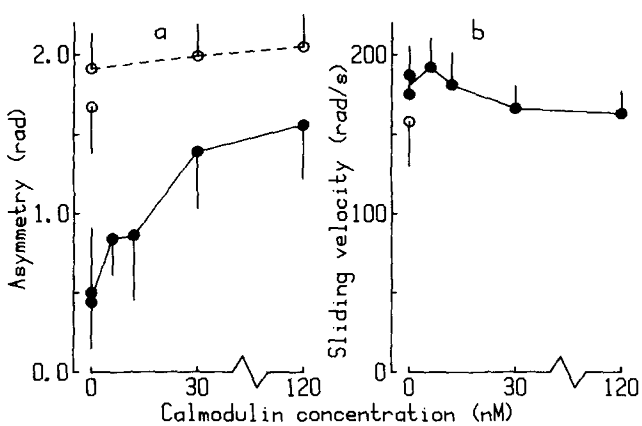

FIGURE 3 (*a*) Effect of bovine brain calmodulin on the asymmetry of flagellar beating of demembranated sea urchin spermatozoa. (○), potentially asymmetric spermatozoa; (●), potentially symmetric spermatozoa, extracted for 20 s with 2.5 mM $CaCl_2$ in the presence of 0.01% Triton. The three points connected by the dashed line are from an experiment with a different sperm sample. Each point represents a sample of 20 spermatozoa, and the standard deviation for each sample is indicated by vertical bars. (*b*) Effect of bovine brain calmodulin on the sliding velocity (proportional to the product of bend angle and beat frequency) during flagellar beating of demembranated sea urchin spermatozoa, from the same experiment shown in Fig. 3*a*.

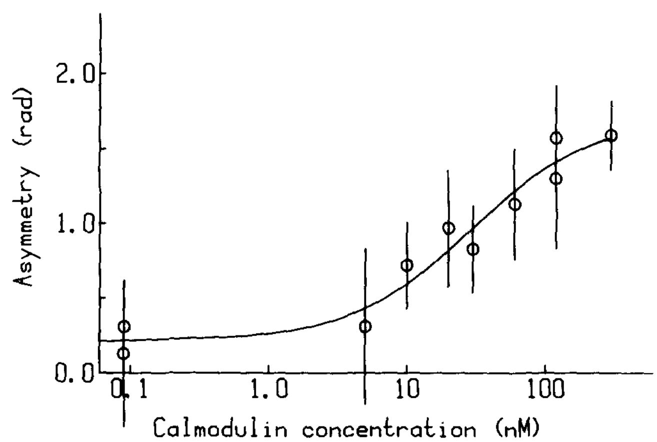

FIGURE 4 Effect of bovine brain calmodulin on flagellar beat asymmetry. Each point represents a sample of 20 spermatozoa, and the standard deviation for each sample is indicated by vertical bars. The curve is obtained by a nonlinear least squares fit to Eq. 1 in the text.

concentration of 2 μg/ml (~120 nM) and half-maximal response is obtained with calmodulin concentrations of 10–40 nM. Fig. 3*a* also shows that there is little response of potentially asymmetric spermatozoa to added calmodulin. After extraction with Triton and millimolar Ca^{++}, the potentially symmetric spermatozoa also have a slightly increased beat frequency and mean bend angle. Fig. 3*b*, which depicts the effects of calmodulin on sliding velocity, shows that these increases are also reversed by the addition of calmodulin to potentially symmetric spermatozoa. However, the effect of calmodulin on sliding velocity is small compared with its effect on asymmetry.

Results from another experiment of this type are shown in Fig. 4, plotted on a logarithmic scale of calmodulin concentrations. The curve has been obtained by a nonlinear least squares fitting procedure by using a three-parameter model given by Eq. 1, where *C* represents calmodulin concentration:

$$\Delta\theta = \Delta\theta_{min} + (\Delta\theta_{max} - \Delta\theta_{min})/(1 + K/C). \qquad (1)$$

The parameters are $\Delta\theta_{min}$, asymmetry in the absence of added calmodulin; $\Delta\theta_{max}$, asymmetry with saturating calmodulin concentrations; and K, calmodulin concentration for 50% saturation of the asymmetry response.

The results shown in Figs. 3 and 4 were obtained with reactivation solutions containing 1 mM EGTA and only the small amounts of Ca^{++} added with the demembranated spermatozoa and the calmodulin samples, ~0.025 mM, so the

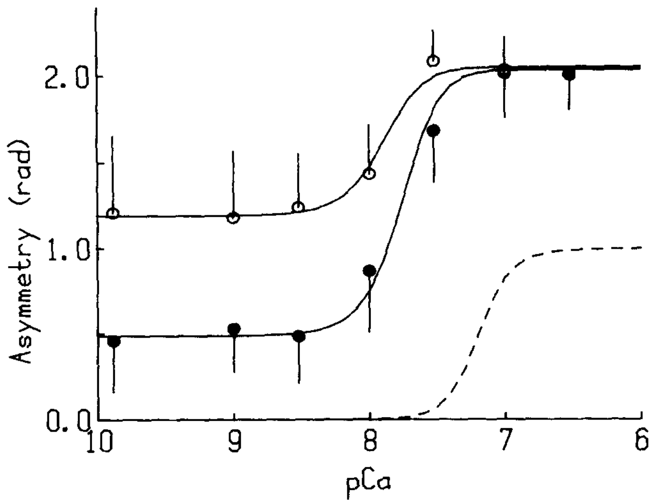

FIGURE 5 Effect of Ca^{++} concentration on flagellar beat asymmetry of potentially symmetric spermatozoa. (●), in the absence of added calmodulin; (○), with 16 nM calmodulin added to the reactivation solution. Each point typically represents the mean value from one or more samples of 20 spermatozoa from each of two different experiments with different sperm samples, and the mean standard deviations for the samples are indicated by vertical bars. Solid lines were obtained from the model for Ca^{++} binding by calmodulin that is discussed in the text; the dashed line shows the binding of calcium to calmodulin, according to this model.

free Ca^{++} concentration can be calculated to be $\sim 10^{-10}$ M. The effect of higher Ca^{++} concentrations on asymmetry is shown in Fig. 5, in the presence and absence of added calmodulin. Although these experiments suggest that calmodulin and Ca^{++} have similar effects on the asymmetry of potentially symmetric flagella, with the asymmetry saturating at similar levels, the situation is actually more complicated. In contrast to the small decrease in mean bend angle that results from the addition of calmodulin, there is a large decrease in the mean bend angle of the flagellar bending patterns at Ca^{++} concentrations $>10^{-8}$ M. The bending patterns show a damping out of the bending waves, so that the bending is compressed into the basal portion of the flagellum. This appears to be characteristic of the response of *Lytechinus* spermatozoa to Ca^{++} (35); it was not seen in earlier work with *Strongylocentrotus* spermatozoa (3).

Fig. 6 shows the results of an experiment in which the duration of the exposure to Triton and millimolar Ca^{++} to produce potentially symmetric spermatozoa was varied over a range of 10 s–5 min. As shown previously (35), the conversion from the potentially asymmetric condition to the potentially symmetric condition is time dependent, but appears to be complete in 30 s or less. The small increase in asymmetry observed after 300 s may be a relatively nonspecific deterioration of the spermatozoa, as it is associated with a decrease in mean bend angle from 2.4 to 2.5 rad for times from 0 to 100 s to 2.3 rad at 300 s. The response of the potentially symmetric spermatozoa to 2 μg/ml calmodulin decreases if the exposure to Triton and millimolar Ca^{++} is extended beyond 30 s. This decrease appears to be dependent on the continued exposure to Triton and Ca^{++}, since spermatozoa extracted for 20 s and then incubated in reactivation solution for 5 min before addition of calmodulin showed a normal response to calmodulin (data not shown). This decrease in response to calmodulin could result from proteolysis of the relevant calmodulin-binding protein(s). However, attempts to prevent the decreased response by addition of various protease inhibitors have been unsuccessful.

Quantitative Analysis of Sperm Calmodulin Activity

These analyses were carried out to obtain information about both the amount of calmodulin that appeared to be extracted during the conversion from potentially asymmetric to potentially symmetric spermatozoa and the relationship of this calmodulin to the amount of calmodulin that remained in the flagellum after extraction with Triton and Ca^{++}. Three methods were used to estimate the calmodulin activity: radioimmunoassay using commercially prepared anti-calmodulin and ^{125}I-labeled calmodulin; comparison of the asymmetry-restoring activity with the asymmetry-restoring activity of brain calmodulin; and comparison of the phosphodiesterase-activating activity with the activity of brain calmodulin. All of these assays were performed with crude extracts, which may contain other components that interfere with an accurate assay of calmodulin, so the results should be considered only rough estimates of calmodulin content.

The assay results are summarized in Table I. The results for samples from whole spermatozoa are expressed as the amount of calmodulin activity corresponding to the 100-μl

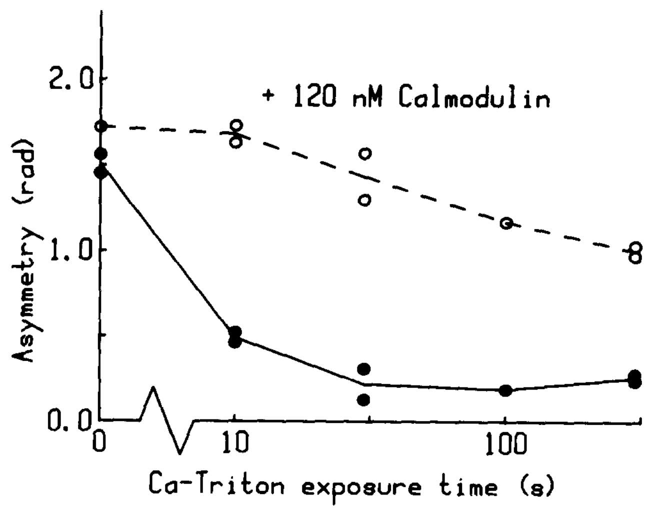

FIGURE 6 Effect of Ca-Triton extraction time on the asymmetry of reactivated movement and the response to calmodulin.

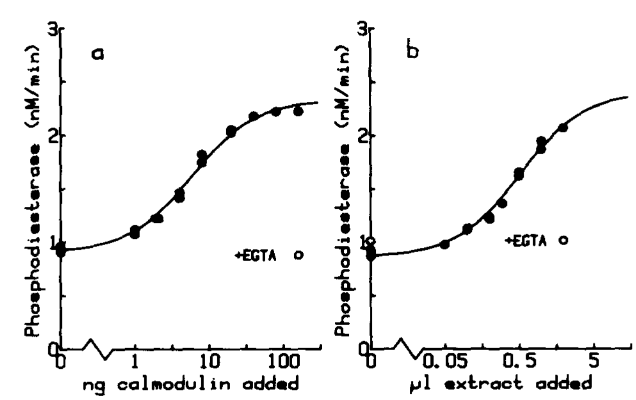

FIGURE 7 Example of a phosphodiesterase assay, showing the activation of phosphodiesterase activity by bovine brain calmodulin (*a*) and by an S3 extract (*b*). (○), the reversal of activation by addition of 0.7 mM EGTA. Curves were obtained from a simple saturation model, using a nonlinear least squares fitting process.

Table I
Calmodulin Assays

Sample	μg Calmodulin measured		
	By RIA	By Δθ	By PDE
100-μl samples of *Lytechinus* spermatozoa containing 4.2 mg protein (*n* = 4; range 3.4–5.0 mg)			
Whole spermatozoa	3.1 (5; 2.2–3.6)*	5.2 (4; 3.7–7.3)	3.5 (4; 2.8–5.3)
Triton-EGTA extract	Not examined	1.4 (2; 1.1–1.7)	2.2 (2; 2.0–2.5)
Pellet after Triton-EGTA extraction	1.2 (4; 1.0–1.5)	1.8 (3; 1.6–2.0)	1.2 (4; 1.0–1.3)
Triton-Ca extract	0.25 (1)	0.15 (2; 0.1–0.2)	0.5 (2; 0.4–0.6)
Pellet after Triton-Ca extraction	1.1 (4; 0.9–1.2)	1.3 (3; 1.0–1.7)	0.45 (4; 0.4–0.6)
Isolated flagella from *Lytechinus* spermatozoa containing 1 mg protein			
Whole flagella	2.5 (2; 2.0–3.0)	Not examined	Not examined
Triton-EGTA extract	1.2 (2; 0.8–1.6)	0.6	0.4
Second Triton-EGTA extract	0.1	<0.01	Not detected
Triton-Ca extract	0.2 (2; 0.14–0.25)	0.17	0.1 (2; 0.05–0.13)
Pellet after Triton-Ca extraction	0.9 (2; 0.4–1.4)	0.5	0.1

RIA, radioimmunoassay; PDE, phosphodiesterase.
* In parenthesis, (*n*; range calmodulin in μg).

samples of diluted spermatozoa that we used, which contained an average of 4.2 mg protein. Previous work on sea urchin spermatozoa indicates that this quantity of sperm protein corresponds to ~2.1×10^9 spermatozoa (17, 30), and that the flagella from these spermatozoa should contain ~1.0 mg protein and 0.5 mg tubulin (6, 13, 17). Therefore, to facilitate comparison, the results of the assays on isolated flagella samples in the lower part of Table I have been converted to a per 1 mg protein basis. An example of the results of a phosphodiesterase activation experiment is shown in Fig. 7.

Additional Observations on Conversion to Potential Symmetry

The conversion of demembranated spermatozoa to the potentially symmetric condition requires the presence of Triton. However, it is not inhibited by 2% polyethylene glycol, which will inhibit the demembranation of flagella by Triton (data not shown). Triton has been shown to bind to calmodulin and inhibit calmodulin activation of phosphodiesterase (40); in fact, it works almost as well as well-known calmodulin-binding drugs such as TFP. This same activity of Triton appears to be involved in the conversion of sperm to the potentially symmetric condition, because TFP is also effective in producing potentially symmetric spermatozoa, as is shown by the experiment summarized in Table II.

To demonstrate the effect of TFP, the Triton concentration must be reduced to a level that is insufficient to support the conversion to the potentially symmetric condition. This is easiest to do, as shown in Table II, by diluting the potentially asymmetric spermatozoa into a reactivation solution containing 1 mM free Ca^{++}. 20 s after the addition of the spermatozoa to this solution, EGTA is added to bring the EGTA concentration to 3 mM and reduce the free Ca^{++} concentration to $<10^{-9}$ M. As shown in Table II, *D* these spermatozoa remain

Table II
Preparation of Potentially Symmetric Flagella with TFP

	Treatment	Δθ
		rad
A	Potentially symmetric spermatozoa (Triton-Ca extracted) added to reactivation solution containing 3 mM EGTA	0.85 ± 0.30
B	Potentially asymmetric spermatozoa added to reactivation solution containing 3 mM EGTA	1.80 ± 0.29
C	Same as in B with 50 μM TFP added	1.83 ± 0.27
D	Potentially asymmetric spermatozoa added to reactivation solution containing 1 mM Ca^{++}, followed by addition of 3 mM EGTA after 20 s	1.67 ± 0.19
E	Same as in D with 50 μM TFP added	0.99 ± 0.47

asymmetric, with a mean Δθ that is almost as high as that obtained with spermatozoa diluted directly into reactivation solution containing EGTA (Table II, *B* and *C*). However, when the reactivation solution also contains 50 μM TFP (Table II, *E*), the asymmetry of flagellar bending is reduced after the 20-s exposure to 1 mM Ca^{++} in the presence of TFP, almost to the level obtained with this sperm preparation after the normal treatment to produce potential symmetry (Table II, *A*). The result in *C* is another control which shows that the TFP has no effect if there is no exposure to high Ca^{++}.

This experiment is also interesting because it shows that the conversion to potential symmetry occurs in the normal time, even if the sperm concentration is only 1% of its usual concentration, as long as the appropriate Ca^{++} and TFP concentrations are maintained. The conversion, therefore, does not appear to be the result of a solubilized enzymatic activity, such as a Ca^{++}-activated protease activity.

DISCUSSION

Calmodulin in Sea Urchin Sperm Flagella

Previous work has provided evidence for the presence of calmodulin in sea urchin spermatozoa. Jones et al. (27) extracted spermatozoa by freezing, thawing, and sonicating them in 1 mM EDTA, 10 mM Tris buffer (pH 8.0) and obtained an extract with phosphodiesterase activating activity equivalent to ~0.2 μg calmodulin/2.1 × 10^9 spermatozoa. Garbers et al. (14), starting with a soluble fraction of homogenized sea urchin spermatozoa, purified calmodulin by ammonium sulfate precipitation, DEAE chromatography, and gel filtration, and confirmed the identification by amino acid analysis and phosphodiesterase activation. Their yield, after several purification steps, was equivalent to 1.7 μg of calmodulin from 2.1 × 10^9 spermatozoa. Our extracts of whole *L. pictus* spermatozoa contain calmodulin-like activity measured by radioimmunoassay equivalent to 3.1 μg calmodulin/2.1 × 10^9 spermatozoa. This includes both the easily solubilized calmodulin in the membrane-matrix fraction, which was probably obtained in the extracts used by previous workers, and also calmodulin tightly bound to the axoneme. The activity obtained by Jones et al. (27) is considerably less than obtained here or by Garbers et al. (14). With other spermatozoa, Jones et al. reported that almost all of the calmodulin activity was associated with head fractions, with none associated with flagellar fractions. The rather mild extraction procedure used by Jones et al. may have selectively extracted calmodulin from the acrosomal region without extracting the flagellar calmodulin.

We have also examined the calmodulin activity of isolated sea urchin sperm flagella and obtained an activity measured by radioimmunoassay equal to ~2.5 μg calmodulin/mg flagellar protein. This is slightly less than the value of 4.0 obtained by quantitative densitometry of calmodulin bands on gels of detergent extracts of gill cilia (41). Our results indicate that most, and possibly almost all, of the calmodulin in the spermatozoa is located in the flagella. 1 mg flagella is expected to contain ~0.5 mg tubulin. A weight ratio of 2.5 μg calmodulin/0.5 mg tubulin corresponds to a molar ratio of 1 calmodulin per 30 tubulin dimers, which is equivalent to 1.3 calmodulin molecules per dynein arm if there are 2 dynein arms per 24 nm along the length of each flagellar doublet microtubule. The uncertainty in this estimate is sufficient to accommodate a stoichiometry of either one or two calmodulin molecules per dynein arm if calmodulin is found to be associated with dynein, as suggested by some reports of calmodulin-dynein interactions (2, 23).

About half of the calmodulin of spermatozoa or flagella is readily solubilized during extraction with Triton in the presence of EGTA, and may be either membrane associated or simply soluble in the cytoplasmic matrix. If this fraction from flagella were entirely in the matrix, it would be equivalent to a free calmodulin concentration of ~50 μM. The readily soluble fraction of whole spermatozoa is somewhat larger, ~2 μg (Table I). After demembranation with Triton and activation with cAMP, during extraction with Triton and Ca^{++} at a 400–500-fold dilution of the original concentrated sperm suspension, this matrix calmodulin fraction will be diluted out to a concentration of ~7 nM. After a further 100-fold dilution when spermatozoa are placed in reactivation solution, the free calmodulin concentration will be diluted to ~0.07 nM. In spite of these low ambient calmodulin concentrations, the potentially asymmetric condition is normally stable unless both Triton and millimolar Ca^{++} are present, and the remainder of the flagellar calmodulin appears to stay firmly attached to the axoneme.

Calmodulin Extraction and the Potentially Symmetric State

When demembranated spermatozoa or flagella are extracted in the presence of Triton and millimolar Ca^{++}, a portion of the axoneme-bound calmodulin is removed. This fraction is ~10% of the flagellar calmodulin under the conditions of our extraction experiments but may be less with the shorter extraction times (20 or 30 s) normally used during reactivation experiments. The presence of calmodulin in these extracts has been demonstrated by various methods, including gel electrophoresis, radioimmunoassay, and phosphodiesterase activating activity.

This extraction with Triton and millimolar Ca^{++} also causes the conversion of the spermatozoa from the potentially asymmetric state to the potentially symmetric state. Their asymmetry can be restored by the concentrated calmodulin-containing extracts or by purified brain calmodulin. The calmodulin content of the extracts, as judged by radioimmunoassay or phosphodiesterase activation, is sufficient to explain the asymmetry-restoring activity of the extracts. However, our results are not precise enough to establish that all of the asymmetry-restoring activity of the extracts is caused by calmodulin and exclude the presence of minor components with asymmetry-restoring activity that binds to the trifluorophenothiazine affinity resin but has low immunoreactivity and no ability to activate phosphodiesterase. Gel electrophoresis of Triton-Ca^{++} extracts does not reveal substantial amounts of other proteins with calcium-dependent mobility, such as the 10-kD calcium-binding protein of *Tetrahymena* cilia (34).

Since the conversion to potentially symmetric spermatozoa requires the presence of Triton or TFP, a simple hypothesis would be that these calmodulin-binding compounds (40) facilitate extraction simply by binding to calmodulin and lowering the free calmodulin concentration. However, this hypothesis fails to explain the high (millimolar) calcium concentrations required for the conversion to the potentially symmetric condition. In addition, since the binding constants of these compounds for calmodulin are in the micromolar range and they are used at concentrations of 50–150 μM, they are unlikely to reduce the calmodulin concentration by more than a factor of 100. Such a reduction, by a dilution into reactivation solution that lowers the free calmodulin concentration to <0.1 nM, cannot produce the potentially symmetric state. Therefore, it seems that these calmodulin-binding compounds must be interacting directly with the axoneme in some way that reduces the affinity for calmodulin. After this extraction, much higher concentrations of calmodulin, in excess of 10 nM, are required to restore asymmetry (Fig. 3).

The simplest interpretation of the appearance of calmodulin in the Triton-Ca extracts and the restoration of asymmetry by calmodulin is that the conversion from the potentially asymmetric condition to the potentially symmetric condition is caused by the removal of some of the axonemal calmodulin. However, we have no evidence that eliminates the possibility that the removal of calmodulin is simply a fortuitous accompaniment of some other process that sensitizes the flagellum to exogenous calmodulin and partially desensitizes the flagellum to Ca^{++}.

Since we believe that intracellular Ca^{++} concentrations in healthy spermatozoa are maintained at submicromolar concentration levels, it has never been clear why spermatozoa that are demembranated and reactivated in the continuous presence of EGTA should be potentially asymmetric and show more asymmetric bending than intact spermatozoa swimming in seawater. One possibility is that the easily extracted calmodulin is normally bound to a membrane-bound calmodulin-binding protein. Dissolution of the membranes with Triton might decrease the calmodulin-binding affinity of this protein, releasing calmodulin that can then bind to sites on the axoneme where it establishes the potentially asymmetric condition. Evidence for membrane-bound proteins that bind calmodulin in the presence of EGTA has been obtained in other systems (21). However, Chun and Gibbons (12) demembranated spermatozoa by osmotic shock without using detergents and found that these spermatozoa were also in the potentially asymmetric condition when they were reactivated. The evidence presented here, that the calmodulin removed during the conversion of the flagella to the potentially symmetric condition is bound with a much higher affinity than is indicated by the response of potentially symmetric flagella to calmodulin, also argues against this interpretation.

An alternative interpretation might be that the calmodulin removed by extraction with Triton and Ca^{++} is a normal constituent of the axoneme, part of its normal mechanism for response to Ca^{++} concentrations. The potentially symmetric state is then regarded as a pathological state that only coincidentally resembles the symmetric bending generated by live spermatozoa. Some other mechanism, such as, for example, a more extensive phosphorylation of control proteins, may be normally responsible for keeping the flagellum of live spermatozoa in a state that generates symmetric bending waves. An analogous situation is seen for the initial activation of motility. Spermatozoa that are not incubated with cAMP to activate their motility can be activated by extraction with Triton and Ca^{++} (4). However, in this case it is clear that the activation by Triton and Ca^{++} produces suboptimal motility (4) and that activation by cAMP-dependent phosphorylation is a more normal mechanism for activation of motility (4).

Modulation of Asymmetry by Calmodulin and Calcium

After extraction with Triton and Ca^{++}, the response of potentially symmetric sperm flagella to exogenous brain calmodulin is consistent with the simple binding equilibrium described by Eq. 1, with no evidence of cooperativity. Similar results are normally seen in other calmodulin-dependent systems (21, 28). This interaction occurs in the presence of EGTA, at free calcium concentrations as low as 10^{-10} M. Although calcium-dependent effects of calmodulin are well known, there are other less well-known situations in which calmodulin interacts with proteins in the presence of EGTA at very low calcium concentrations. The adenylate cyclase of *Bordetella pertussis* is activated by calmodulin in the presence of EGTA with a calmodulin concentration for half-maximal activation of 24 nM (21), similar to the values obtained for half-maximal restoration of the asymmetry of potentially symmetric flagella (e.g., Figs. 3 and 4). In this case, the calmodulin concentration for half-maximal activation by calmodulin is lowered to 0.1 nM in the presence of 90 μM Ca^{++} (21). Calcium-independent binding of calmodulin to troponin I at high calmodulin concentrations has also been reported, as have other situations in which calmodulin appears to bind in the absence of Ca^{++} or independently of Ca^{++} (1, 19, 24, 30, 43, 48).

Ca^{++}-independent calmodulin actions that require calmodulin concentrations several orders of magnitude higher than those required in the presence of Ca^{++} indicate that in the presence of appropriate receptor proteins, calmodulin normally exists as an equilibrium mixture containing a small fraction of the molecules in the conformation that have the biological activity of interest. If the active conformation has a higher affinity for Ca^{++} binding than the inactive conformation, binding of Ca^{++} will then shift this equilibrium towards the active conformation. This model (47) appears applicable to the asymmetry-restoring effects of calmodulin on flagella, as well as to troponin I binding and the activation of the *B. pertussis* adenylate cyclase. The results in Fig. 5 confirm this interpretation. The alternative interpretation, that even at very low ($\sim10^{-10}$ M) Ca^{++} concentration the active calmodulin is the very small fraction of the calmodulin that has bound Ca^{++}, would predict that the asymmetry would increase with increasing Ca^{++} concentration even at very low Ca^{++} concentration; this is not seen in Fig. 5.

The response of potentially symmetric sperm flagella to exogenous calmodulin does not answer directly the more fundamental question about flagellar calmodulin: Is the flagellar calmodulin (especially the fraction remaining in the axonemes of potentially symmetric flagella) responsible for mediating the increase in asymmetry that is caused by increasing Ca^{++} concentrations? The response to Ca^{++} shown in Fig. 5 occurs at Ca^{++} concentrations that are one or two orders of magnitude lower than those usually associated with Ca^{++}-calmodulin activation.

In interpreting these curves, it should be remembered that the axoneme is a complex, integrated, system in which there may be large-scale cooperative interactions, in contrast to the situation existing, for instance, in a solution of independent phosphodiesterase molecules. The saturation responses seen when calmodulin or Ca^{++} concentrations are increased may not therefore indicate saturation of ligand binding but only a saturation of the asymmetry response of the axonemes. This saturation may occur even if only a small fraction of the axonemal calmodulin binds Ca^{++} and then interacts in a Ca^{++}-dependent manner with a calmodulin receptor protein.

Using Eq. 1 to develop this line of reasoning, we redefine $\Delta\theta_{min}$ as intrinsic asymmetry of the flagellum in the absence of any effects of calmodulin, $\Delta\theta_{max}$ as maximum asymmetry that can be produced by calcium and calmodulin effects, C as concentration of calmodulin in the active form, and K as the concentration of calmodulin in the active form that gives a half-maximal response.

$$C = F(C_I + C_E) \tag{2}$$

where C_E is the concentration of calmodulin in the reactivation solution, C_I is the effective concentration of calmodulin intrinsic to the flagellum, and F is the fraction of the calmodulin in the active form. We assume that the external and internal calmodulin components have similar Ca^{++}-binding behavior. For a model in which calmodulin binds four Ca^{++} ions (47), the fraction of calmodulin in the active form is given by

$$F = 1/(1 + A/K_cB), \tag{3}$$

where K_e is the equilibrium constant for formation of the active form of calmodulin in the absence of Ca^{++}, and A and B are functions of the Ca^{++} concentration, S, given by

$$A = 1 + K_1S\{1 + K_2S[1 + K_3S(1 + K_4S)]\}, \text{ and}$$
$$B = 1 + K_1'S\{1 + K_2'S[1 + K_3'S(1 + K_4'S)]\}. \quad (4)$$

The affinity constants for binding of Ca^{++} to the four binding sites on the inactive form of calmodulin are K_1, K_2, K_3, and K_4 and for binding to the active form of calmodulin are K_1', K_2', K_3', K_4' (47). The curves fitted to the data in Fig. 6 have been calculated by using $\Delta\theta_{min} = 0.0$ rad, $\Delta\theta_{max} = 2.05$ rad, $C_I = 4.5$ nM, $K = 0.01\ C_I$, $K_e = 0.003$, $K_n = 1 \times 10^5\ M^{-1}$, and $K_n' = 6.5 \times 10^7\ M^{-1}$. Also shown in Fig. 5 is a curve for the saturation of the calcium-binding sites on the calmodulin as a function of Ca^{++} concentration. This curve can be shifted towards somewhat higher Ca^{++} concentrations by the use of smaller values for K and K_e while their ratio is kept constant.

The data in Fig. 5 are therefore consistent with a mechanism for response to Ca^{++} that is mediated by calmodulin, with half-maximal response occurring when only 1% of the intrinsic calmodulin of potentially symmetric flagella is in the active conformation. However, this model does not explain the high Ca^{++} sensitivity of the flagella without also assuming that the active conformation of calmodulin, presumably the form that is interacting with a receptor in the axoneme, has a Ca^{++} binding affinity considerably greater than the Ca^{++} binding affinity of free calmodulin (28).

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