Identification, Characterization, and Functional Correlation of Calmodulin-dependent Protein Phosphatase in Sperm

J. S. Tash,* M. Krinks,‡ J. Patel,* R. L. Means,* C. B. Klee,‡ and A. R. Means*

*Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030; and ‡National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Abstract. Preliminary data demonstrated that the inhibition of reactivated sperm motility by calcium was correlated with inhibited protein phosphorylation. The inhibition of phosphorylation by Ca^{2+} was found to be catalyzed by the calmodulin-dependent protein phosphatase (calcineurin). Sperm from dog, pig, and sea urchin contain both the Ca^{2+}-binding B subunit of the enzyme (M_r 15,000) and the calmodulin-binding A subunit with an M_r of 63,000. The sperm A subunit is slightly higher in M_r than reported for other tissues. Inhibition of endogenous calmodulin-dependent protein phosphatase activity with a monospecific antibody revealed the presence of 14 phosphoprotein substrates in sperm for this enzyme. The enzyme was localized to both the flagellum and the postacrosomal region of the sperm head. The flagellar phosphatase activity was quantitatively extracted with 0.6 M KCl from isolated flagella from dog, pig, and sea urchin sperm. All salt-extractable phosphatase activity was inhibited with antibodies against the authentic enzyme. Preincubation of sperm models with the purified phosphatase stimulated curvolinear velocity and lateral head amplitude (important components of hyperactivated swimming patterns) and inhibited beat cross frequency suggesting a role for this enzyme in axonemal function. Our results suggest that calmodulin-dependent protein phosphatase plays a major role in the calcium-dependent regulation of flagellar motility.

THE apparent requirement of cAMP-dependent protein phosphorylation for flagellar motility (Tash et al., 1984, 1986) is contrasted by the inhibitory and waveform modulating effects of Ca^{2+} (Hyams and Borisy, 1978; Gibbons, 1980). Considerable effort has been exerted by a number of laboratories concerning possible mechanisms of action of Ca^{2+} on the regulation of flagellar movement (Satir, 1985; Gibbons, 1982; Brokaw et al., 1974; Bessen et al., 1980). More recent attention has concentrated on calmodulin as the potential primary signal transducer in Ca^{2+}-regulated flagellar motility. However, results have been contradictory, which may result from differences in species as well as the techniques used (Gitelman and Witman, 1980; Blum et al., 1980). Perhaps the most compelling evidence to date to suggest an involvement of calmodulin in this regulation comes from the observations of Brokaw and Nagayama (1985). The asymmetry-inducing effects of calmodulin-containing flagellar extracts were eliminated by passing the extracts through trifluoperazine columns. Furthermore, they found that purified calmodulin reverses the symmetric swimming patterns of sperm demembranated in the presence of Ca^{2+} to produce asymmetric flagellar bending. While evidence suggesting an involvement of calmodulin in the regulation of flagellar motility is increasing, a defined calmodulin-dependent biochemical pathway, which could explain the effects of Ca^{2+}–calmodulin on motility, remains to be elucidated and characterized.

The present study reports the identification of calmodulin-dependent protein phosphatase (calcineurin, EC 3.1.3.16) in dog, pig, and sea urchin sperm by four different criteria. In addition, 14 substrates for the calmodulin-dependent phosphatase have been identified in sperm. Preliminary analysis suggests that both phosphatase and calmodulin remain associated with dynein after sucrose gradient sedimentation. Exogenous calmodulin-dependent phosphatase was found to modify sperm motility in a manner similar to that produced by Ca^{2+} without added phosphatase.

Materials and Methods

Materials

All reagents were ACS grade where available. [γ-^{32}P]ATP (5,000 ci/mmol) was obtained from ICN Laboratories, Inc., Plainview, NY. Affinity-purified antitubulin was obtained from Dr. B. R. Brinkley (University of Alabama, Birmingham, AL). Affinity-purified anti-apoVLDL-II was obtained from Dr. L. Chan (Baylor College of Medicine, Houston, TX).

Preparation of Sperm and Sperm Fractions

Sperm for use as live preparations were prepared from dog ejaculates in Ca^{2+}-free Krebs' Ringer phosphate buffer as described previously (Tash and Means, 1982). To minimize sperm sticking to the coverglass and microscope slide surfaces during motility analysis, BSA (Sigma Chemical Co., St. Louis, MO) was added to the initial sperm wash buffers to a final concentration of 2.5% (wt/vol), and slides and coverslips were coated using a 1% (wt/vol) solution of cellulose nitrate in methanol (Chapeau and Gagnon, 1987) and then coated with 2.5% BSA followed by washing with H_2O.

Dog sperm for reactivation were prepared from ejaculates as above, suspended in Hepes reactivation buffer (10 mM Hepes, 2 mM EGTA, 1 mM dithiothreitol [DTT], 5 mM $MgSO_4$, 100 mM NaCl, pH 7.5) as described previously (Tash et al., 1984), and modified with 1 mg/ml soybean trypsin inhibitor (T-9003; Sigma Chemical Co.). Sperm were permeabilized by the addition of NP-40 (BDH Chemicals, Ltd., Poole, England) to a final concentration of 0.015% (vol/vol). In preincubation experiments, EGTA was replaced with 0.1 mM BAPTA[1] (1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*',*N*'-tetraacetic acid; Molecular Probes, Eugene, OR) to allow adequate buffering of Ca^{2+} during subsequent reactivation.

Porcine sperm were collected from a Yorkshire boar using a collection dummy. The filtered sperm-rich fraction was centrifuged for 10 min at 800 *g* to remove seminal plasma before washing by the same method used for dog sperm described above.

Sea urchin (*S. purpuratus*) sperm were collected by removing the mouth and filling the body cavity with 0.5 M KCl. Sperm were collected in artificial sea water and washed twice in artificial sea water before processing for flagellar isolation and dynein extraction as described below.

Flagellar Reactivation

Reactivation of permeabilized dog sperm was achieved by the addition of the following to give the indicated final concentration (in parentheses): ATP (100 μM), cAMP (0.5 μM), and, when required, $CaCl_2$ (1×10^{-8} to 1×10^{-4} M free cation concentration). The required amount of Ca^{2+} to give the desired free Ca^{2+} concentration was calculated by the algorithms published by Goldstein (1979). The samples were then warmed to 37°C for 3 min to reactivate flagella.

Digital Image Analysis of Reactivated Sperm Motility

The curvolinear velocity, straight line velocity, linearity, mean lateral head displacement, beat cross frequency, and mean radius of curvative of trajectories were measured in reactivated sperm using the CellSoft real time digital image processing system (Version 3.51c, CryoResources Ltd., New York, NY). These parameters have been defined by Mack et al. (1988) and Robertson, L., D. P. Wolf, and J. S. Tash (manuscript submitted for publication). A chamber depth of 32 μm was used. CellSoft setup parameters were as follows: number of frames to analyze, ≥13; data acquisition rate, 30 frames/s; minimum sampling for motility and Brownian motion filter, 4 points; minimum sampling for velocity, 13 points; maximum voelocity, 400 μm/s; minimum velocity, 10 μm/s. For lateral head displacement and beat cross frequency parameters the following values were set: minimum number of points, 13; minimum velocity, 10 μm/s; minimum linearity, 0. For circular motion parameters the following values were set: minimum number of points, 13; minimum velocity, 10 μm/s; maximum radius, 200 μm. All parameters were measured within the first 3 min of reactivation and the mean value was calculated from all individual cell measurements at each Ca^{2+} concentration. Statistics for each parameter were calculated from the individual measurements for each motile cell (40–80 motile cells were measured for each point).

Protein Phosphorylation

Protein phosphorylation was carried out under the same conditions used for reactivation except that ATP was replaced with [γ-^{32}P]ATP at (1,000–2,000 cpm/pmol). Phosphorylation was allowed to proceed for 2 min at 37°C, then terminated by the addition of 2-[*N*-cyclohexylamino]-ethanesulfonic acid–SDS sample buffer (Tash et al., 1986).

The effect of anticalcineurin antibodies on protein phosphorylation was examined by preparing permeabilized sperm as described above. The sperm were then incubated overnight in an ice bath in the presence of 167 μg/ml anticalcineurin antibodies in the absence of reducing agents. This level of antibody was used based on its ability to inhibit endogenous sperm calmodulin-dependent phosphatase activity by biochemical determinations. Phosphorylation was then initiated by the addition of [γ-^{32}P]ATP and cAMP and/or Ca^{2+} as described above. Phosphorylation was allowed to proceed for 2 min at 37°C before preparation for high resolution two-dimensional PAGE.

Phosphatase Assay

Calmodulin-dependent phosphatase activity was measured using *p*-nitrophenylphosphate (p-NPP) as a substrate by a modification of the method of Pallen and Wang (1983). Enzyme samples (1–25 μl) were mixed with 100 μl of stock assay buffer (40 mM Tris-HCl, 0.2 M NaCl, 1 mM DTT, 0.2 mg/ml BSA, 3 mM $CaCl_2$, 12 mM $MgCl_2$, pH 8.0), 1 μl calmodulin (1 mg/ml), 10 μl of 100 mM p-NPP, and H_2O to a total assay volume of 200 μl. To determine protein phosphatase activity in the absence of Ca^{2+}, EGTA was included in the assay mixture to a final concentration of 8.2 mM to reduce free Ca^{2+} to 6.4 nM. Dynein activity ATPase was inhibited by including 25 μM orthovanadate. p-NPP hydrolysis at 20°C was monitored continuously at 400 nm in a spectrometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) with a chart recorder. Rates were determined from the linear portion of the tracings using ε^{M}_{400nm}p-NPP = 15,300.

High Resolution Two-dimensional PAGE

Protein phosphorylation/dephosphorylation was analyzed by high resolution two-demensional PAGE using the Health Products MegaDalt multiple gel casting system based on the method of Anderson and Anderson (1978*a*, *b*), as used previously in this laboratory (Tash et al., 1984, 1986). The first dimension was disc IEF using pH 3.5–10 ampholines (a 2:1 mixture of LKB and Pharmacia wide-range ampholines, respectively). The second dimension was SDS-PAGE using a 10–20% acrylamide gradient.

Gels were stained using the silver-based color stain of Sammons et al. (1981), then dried and autoradiographed using Kodak XS-5 film and Quanta III intensifying screens. Autoradiographic signals were quantitated by digital image processing by the method of Mariash et al. (1982).

Indirect Immunofluorescence

Indirect immunofluorescence of calmodulin-dependent protein phosphatase in sperm was performed by a previously published procedure (Tash and Means, 1982). Nonspecific antibody binding was blocked using 5% (wt/vol) nonfat dry milk. Sperm were permeabilized with 0.01% Triton X-100 after fixation. The primary antibody was affinity-purified rabbit anti-bovine brain calcineurin at 150 μg/ml. The second antibody was fluorescein-tagged goat anti-rabbit IgG (Miles Laboratories Inc., Naperville, IL) at 1:50 dilution. As a control, anticalcineurin was incubated overnight at 0°C with fivefold molar excess calcineurin and then used on replicate cover glasses as above.

"Western" Immunoblot Analysis

Washed sperm were prepared as described above and run on 5–15% SDS–polyacrylamide gradient slab gels. Proteins were transferred by electrophoresis to nitrocellulose sheets by a modification (Guerriero et al., 1981) of the method of Towbin et al. (1979). The nitrocellulose sheet was blocked after transfer with 5% (wt/vol) nonfat dry milk (Johnson et al., 1984) for 18 h at 4°C. Antigen was localized with affinity-purified rabbit anti-bovine brain calcineurin at 1.4 ng/ml and [^{125}I]protein A followed by autoradiography.

Crude Dynein Preparation

Dynein containing high-salt extracts was prepared from sea urchin sperm by the 20% sucrose method of Bell et al. (1982). All buffers were prepared without Ca^{2+}. High-salt extracts from dog and pig sperm were prepared by essentially the same method with the following modifications. Sperm were washed in Ca^{2+}-free Krebs' ringer phosphate and then suspended in hypotonic Hepes buffer (10 mM Hepes, 1 mM DTT, 2 mM EGTA, 5 mM $MgSO_4$, pH 7.5). Homogenization was achieved using a teflon glass homogenizer with a clearance of 0.001 in. Heads and whole flagella were separated at 424 *g*. Once the flagella were isolated, salt extraction was carried out by the same method described above for sea urchin.

Calcineurin and Calcineurin Antibodies

Calcineurin was purified to apparent homogeneity from bovine brain as described by Klee et al. (1983). PAGE in the presence of SDS revealed only the two subunits of the enzyme. The enzyme used in these studies was stimulated 10–15-fold by calmodulin. Calmodulin was not detectable in the purified enzyme and the basal activity measured in the absence of calmodulin was not inhibited by phenothiazines but was inhibited by the antibodies described below. With respect to possible contamination with proteases, the enzyme is extremely susceptible to proteolytic activation (Manalan and Klee, 1983), yet no loss of calmodulin stimulation or degradation was evident after incubation for 1 h at 30°C (Klee, C. B., unpublished observation).

1. *Abbreviations used in this paper*: BAPTA, 1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*',*N*'-tetraacetic acid; p-NPP, *p*-nitrophenylphosphate.

Table I. Ca^{2+}-dependent Phosphatase Activity in Sperm Fractions

Species	Assay	Whole sperm	Flagella	High-salt extract	Extracted flagella
Canine	$+Ca^{2+}$	121.0	10.4*	102	0.0
	$-Ca^{2+}$	0	6.7*	73.2	0.0
Porcine	$+Ca^{2+}$	13.0	24.4	44.8*	1.0*
	$-Ca^{2+}$	10.5	0	14.7*	0.0
Sea urchin	$+Ca^{2+}$	32.0*	35.4*	39.8*	1.9*
	$-Ca^{2+}$	22.0*	21.6*	10.5*	0.7*

Flagella were isolated from demembranated washed dog, pig, and sea urchin sperm. The isolated flagella were extracted with 0.6 M NaCl and centrifuged to obtain the high-salt extract and extracted flagella. These fractions as well as homogenized whole sperm were then assayed for Ca^{2+}-dependent phosphatase activity using the p-NPP assay method for calmodulin-dependent protein phosphatase as described in Materials and Methods. Phosphatase assays were performed in the presence of 25 μM ortho-vanadate to inhibit dynein ATPase activity. Phosphatase activity measured in the absence of Ca^{2+} was accomplished by the addition of EGTA to yield a final free Ca^{2+} concentration of 6.4 nM. All activities are expressed as μmol p-NPP cleaved/min per mg protein.
* Assays are means of two separate preparations.

The specific activity of the enzyme, using a synthetic phosphopeptide substrate in the presence of Ca^{2+} (Blumenthal et al., 1986), was 1.3 mol phosphate cleaved/min per mg protein.

Polyclonal antibodies to calcineurin were raised in rabbits using the purified enzyme (Krinks, N. H., A. S. Manalan, and C. B. Klee, manuscript in preparation). An IgG fraction was prepared from the serum by three successive ammonium sulfate precipitations. For immunofluorescence, anticalcineurin was affinity-purified on a calcineurin–Sepharose column. The protease content of the anticalcineurin preparations was not tested directly, however, calcineurin purified on an antibody–Sepharose column yielded undegraded enzyme. By Western immunoblot, the antibody recognized only the two subunits of calcineurin in crude brain extracts (61 and 15 kD). The antibody did not inhibit purified calmodulin-dependent phosphodiesterase or calmodulin-dependent protein kinase II.

Results

This work was stimulated by preliminary studies (Tash, J. S., P. Kelly, and A. R. Means, unpublished observations) indicating that the presence of 1 μM Ca^{2+} during flagellar reactivation modified the phosphorylation of several sperm proteins. Furthermore, the modifications produced by Ca^{2+} could be reversed in many cases, by the anticalmodulin drug, W13. In relative magnitude, the major effect of Ca^{2+} was an inhibition of protein phosphorylation. These preliminary observations suggested that Ca^{2+}–calmodulin-dependent protein phosphatase may be present in sperm.

Distribution of Calcium-dependent Phosphatase in Sperm from Different Species

The subcellular distribution of calcium-dependent phosphatase activity in flagella was examined in canine, porcine, and sea urchin sperm (Table I). Phosphatase activity was determined using conditions optimal for the calmodulin-dependent protein phosphatase (see Materials and Methods). In whole sperm, the phosphatase activity preferred the presence of Ca^{2+}. The lower degree of Ca^{2+} dependence of the enzyme activity in whole porcine and sea urchin sperm may be due to the presence of Ca^{2+}-independent phosphatases that may cleave the substrate under these assay conditions (Srivastava et al., 1982; Singer et al., 1980). Isolated flagella from all three species also contained Ca^{2+}-dependent phosphatase activity. In dog sperm, the specific activity of the phosphatase was lower in flagella, whereas in pig sperm the specific activity was higher, and in sea urchin there was little change. High-salt extraction quantitatively removed phosphatase activity from the flagella of all three species. The large fluctuation in specific activity of the dog sperm preparations is probably a reflection of the small amounts of dog material that were available for these determinations. In terms of flagellar phosphatase activity, the highest specific activity for the enzyme was in the high-salt extract for all three species.

Immunoblot Identification of the Calmodulin-dependent Protein Phosphatase in Sperm

The only enzyme known to catalyze protein dephosphorylation in a Ca^{2+}-dependent manner is the calmodulin-dependent phosphatase (calcineurin). The presence of this enzyme in sperm was investigated by immunoblot analysis (Fig. 1). Monospecific anticalcineurin recognized the purified authentic bovine brain antigen (*Cn*) at M_r 61,000 (A subunit) and the light chain (B subunit) at M_r 15,000. A similar pattern was noted in dog sperm (*Sp*). The A subunit in sperm displayed a slightly higher M_r of 63,000. Additional analysis demonstrated calcium-dependent [^{125}I]calmodulin binding to a sperm protein at 63,000 by the method of Hubbard, M., M. Krinks, and C. B. Klee (manuscript in preparation) and [$^{45}Ca^{2+}$]binding to a 15-kD sperm peptide by the method of Maruyama et al. (1984). The same size subunits (63 and

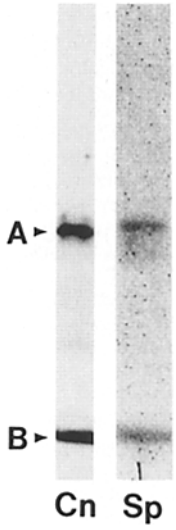

Figure 1. Immunoblot analysis of calmodulin-dependent protein phosphatase in dog sperm. Calcineurin was identified by immunoblot analysis. The left lane represents purified bovine brain calcineurin (*Cn*) and the right lane represents sperm proteins (*Sp*) analyzed by the immunoblot procedure. *A* designates the migration of the A subunit of calcineurin (M_r 61,000). *B* designates the migration of the B subunit of bovine brain calcineurin (M_r 15,000). The sperm A subunit consistently migrated with a slightly higher molecular weight at 63,000.

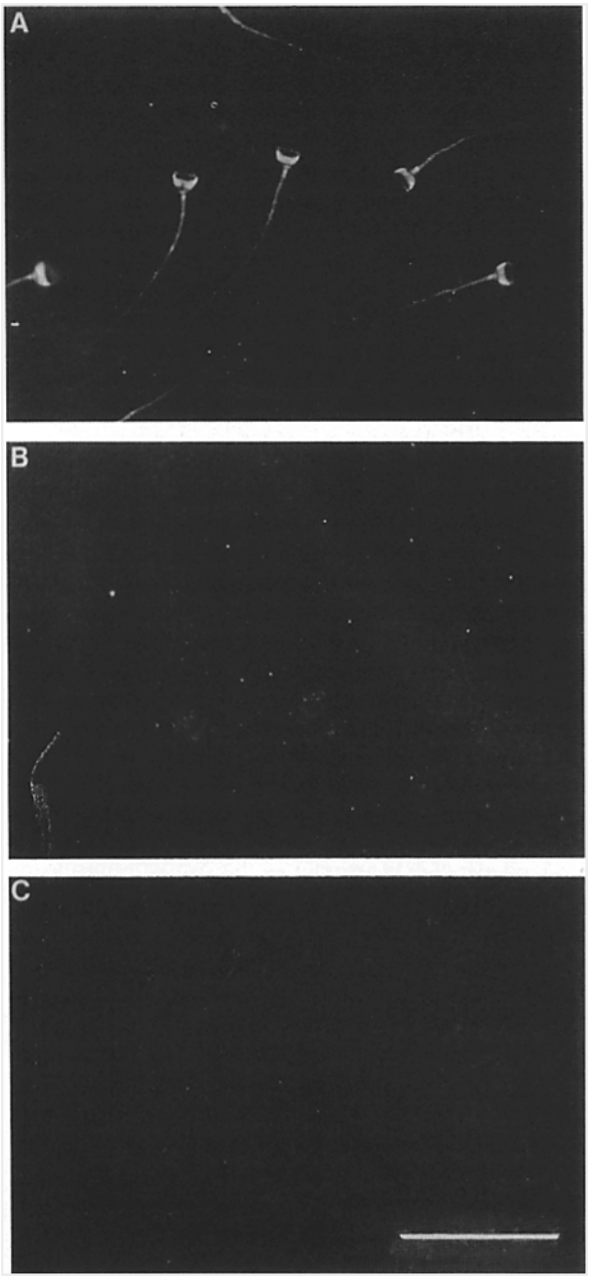

Figure 2. Localization of calcineurin in sperm by indirect immunofluorescence. Calcineurin was localized in dog sperm by indirect immunofluorescence. (*A*) Fluorescence using anticalcineurin at 167 μg/ml. (*B*) Control using anticalcineurin preabsorbed with excess calcineurin. (*C*) Control using second antibody alone. Bar, 25 μm.

15 kD) for the enzyme were present in all three species as assessed by immunoblotting (data not shown).

Indirect Immunofluorescent Localization of Calcineurin in Sperm

The localization of the calcineurin-like antigen in dog sperm was determined by indirect immunofluorescence microscopy (Fig. 2). Staining was noted throughout the flagellum as well as in a narrow band above and a broad band below the equatorial segment in the sperm head (Fig. 2 *A*). Neither primary antibody preincubated with excess antigen (Fig. 2 *B*) nor second antibody alone (Fig. 2 *C*) resulted in a fluorescence signal in the sperm cells. The presence of the enzyme in the sperm head is consistent with the ranges in specific activity noted in the fractionation experiments (Table I).

Anticalcineurin Blocks Ca^{2+}-dependent Dephosphorylation

To determine if the endogenous sperm calmodulin-dependent protein phosphatase affected sperm protein phosphorylation, we used anticalcineurin to block the endogenous phosphatase activity. Permeabilized sperm were incubated overnight at 0°C in the presence of anticalcineurin antibodies. The sperm were then incubated for 2 min under reactivating conditions with [^{32}P]ATP in the presence and absence of cAMP and/or Ca^{2+}, and analyzed by high resolution two-dimensional PAGE and autoradiography. The 2-min incubation was chosen since this is saturating with respect to occupancy of cAMP-dependent phosphorylation sites. The effects of antibody on protein phosphorylation were most evident in the presence of both cAMP and Ca^{2+} (Fig. 3). As indicated by the arrows, 14 peptides (including axokinin, the major 56-kD cAMP-dependent phosphoprotein) were affected by the addition of anticalcineurin. Three major classes of protein phosphorylation were revealed by antibody addition: (*a*) phosphoproteins that were absent in Ca^{2+} but revealed after antibody addition (all marked peptides except axokinin and peptides 3, 8, 10, and 13); (*b*) phosphoproteins that were present in Ca^{2+} but increased by the addition of antibody (axokinin and peptides 3 and 10); and (*c*) phosphoproteins that were inhibited by the addition of antibody (peptides 8 and 13).

Phosphorylation of the Major 56-kD Phosphoprotein Is Modified by Calmodulin-dependent Protein Phosphatase

Analysis of the phosphorylation of the major sperm 56-kD phosphoprotein (axokinin) by digital image processing (Table II) revealed that the Ca^{2+}-dependent increase in phosphorylation produced by anticalcineurin was additive with that produced by cAMP. Identical results were obtained for peptides 3 and 10. Axokinin and peptides 3 and 10 were the only peptides whose phosphorylation was increased twofold in the presence of Ca^{2+} when anticalcineurin was added. As controls for the antibody addition experiment, parallel incubations were carried out using antibodies to tubulin (as an example of an antigen present in sperm) and to apoVLDL-II (an antigen not present in sperm). As summarized in Table II, neither of these antibodies had any demonstrable effect on sperm protein phosphorylation under any of the experimental conditions tested.

Further Characterization of the Enzyme in the High-Salt Extract

Two criteria were used to determine the contribution of calmodulin-dependent phosphatase to the total measurable phosphatase activity in the high-salt extract. SK1 (the synthetic peptide representing the calmodulin-binding fragment of skeletal muscle myosin light chain kinase; Blumenthal et al., 1985) was tested for its ability to inhibit phosphatase activity in the high-salt extract of sea urchin sperm (Fig. 4).

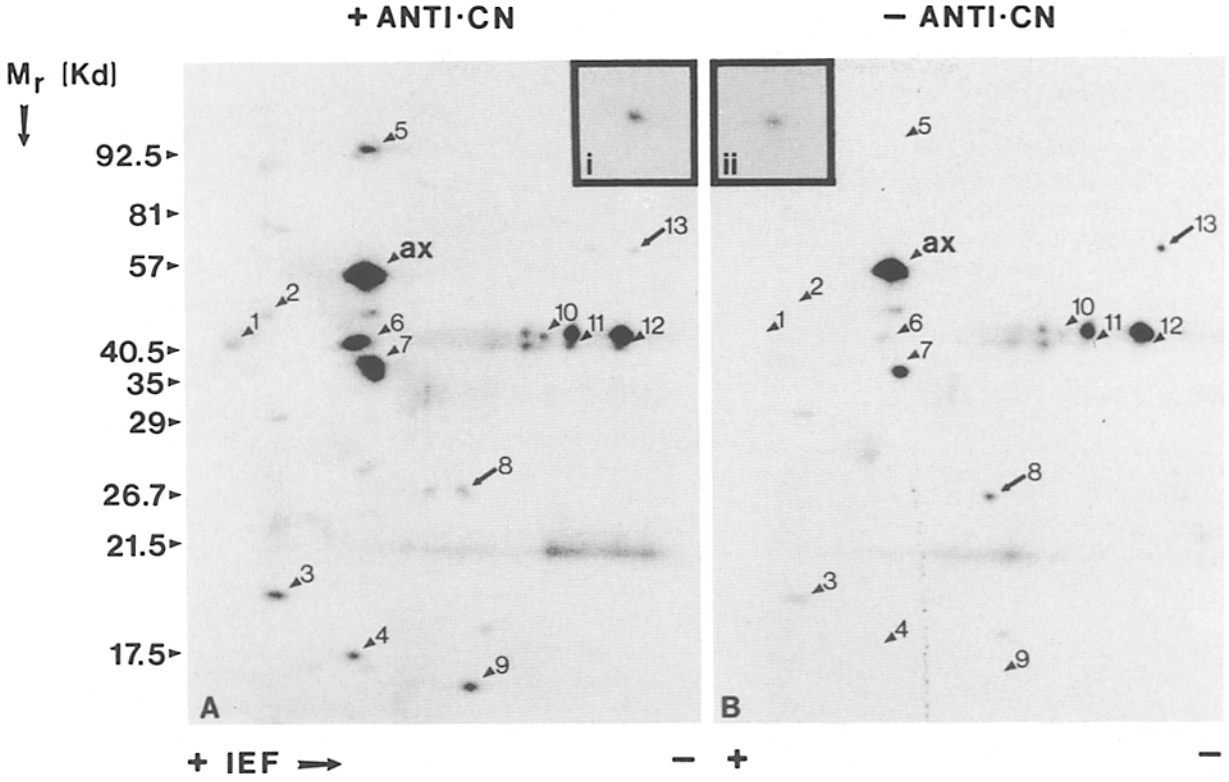

Figure 3. Effect of anticalcineurin on protein phosphorylation. Permeabilized dog sperm were incubated overnight in the presence (+ *ANTI·CN*) or absence (− *ANTI·CN*) of anticalcineurin. The sperm were then incubated for 2 min with [^{32}P]ATP in the presence of cAMP and Ca^{2+}. Resulting phosphorylations were analyzed by high resolution two-dimensional PAGE and autoradiography. The panels were intentionally overexposed to enhance the signals of the minor phosphorylations. The insets (*i* and *ii*) depict the nonoverexposed signals for the axokinin containing region from the same gels.

The high-salt extract used in these experiments showed a two- to threefold stimulation in phosphatase activity in the presence of Ca^{2+}. SK1 inhibited the Ca^{2+}-dependent phosphatase activity in the extracts with an apparent K_I of 1.3 μM. Since not all the activity was Ca^{2+} dependent and was not inhibited by SK1, it was possible that the remaining phosphatase activity was due to another enzyme. This possibility was explored by assaying the enzyme after incubating the high-salt extract in the presence and absence of antibodies to calcineurin. The antibody produced 86 and 100% inhibition of the phosphatase activity measured in the presence and absence of Ca^{2+}, respectively. Thus all of the phosphatase activity in the high-salt extract could be attributed to the calmodulin-dependent phosphatase. Similar results were obtained with *Lytechinus pictus*, dog, and pig sperm high-salt extracts in that the Ca^{2+}-dependent activity was blocked by SK1 and all remaining activity was inhibited with anticalcineurin.

Effect of Ca^{2+} on Motility of Reactivated Dog Sperm

The bidirectional effect of Ca^{2+} on sperm protein phosphorylation suggested that Ca^{2+} may regulate multiple parameters of sperm movement. This possibility was examined by determining the effect of 5×10^{-8} to 5×10^{-4} M free Ca^{2+} on motility of dog sperm reactivated with ATP and

Table II. Quantitative Analysis of the Effect of Anticalcineurin on Axokinin Phosphorylation

Phosphorylation conditions	^{32}P incorporated			
	No antibody	Anti-calcineurin	Anti-tubulin	Anti-apoVLDL-II
Control	6,300	6,500	6,200	6,100
cAMP	10,300	10,300	12,600	8,200
Ca^{2+}	3,800	10,800	5,400	4,400
cAMP + Ca^{2+}	13,800	23,500	9,400	10,400

Permeabilized dog sperm were incubated overnight at 0°C in the presence or absence of rabbit anti-bovine brain calcineurin, rabbit anti-bovine brain tubulin, or rabbit anti-apoVLDL-II. All antibodies were used at a final concentration of 167 μg/ml. This concentration of antibody was used to ensure complete inhibition of the abundant phosphatase activity as determined by biochemical assay of antibody-inhibited phosphatase activity. Sperm were then incubated under reactivation conditions for 2 min with [^{32}P]ATP, in the absence and/or presence of 5×10^{-7} M cAMP or 1×10^{-6} M free Ca^{2+}, as described in the text. Reactions were terminated and analyzed by high resolution two-dimensional PAGE, autoradiography, and digital image processing of the autoradiographs. ^{32}P incorporation is expressed as total counts accumulated into axokinin during the autoradiographic exposure. All gels were exposed for the same amount of time. These results represent a single experiment but replicate experiments gave comparable results.

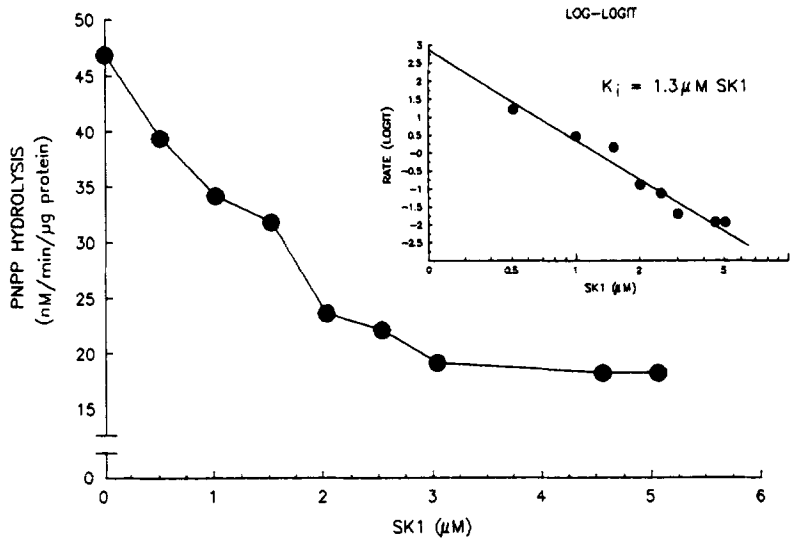

Figure 4. Inhibition of phosphatase activity in sea urchin sperm flagellar high-salt extract by SK1. The high-salt extract of isolated sea urchin sperm flagella was assayed for calmodulin-dependent protein phosphatase activity in the presence of increasing levels of SK1 (Blumenthal et al., 1985; sequence= LKKYLMKRRWKKNFIAVSAANRFKKI) using a standard assay with p-NPP as substrate. To circumvent possible substrate cleavage by dynein ATPase, all assays were performed in the presence of 25 μM orthovanadate.

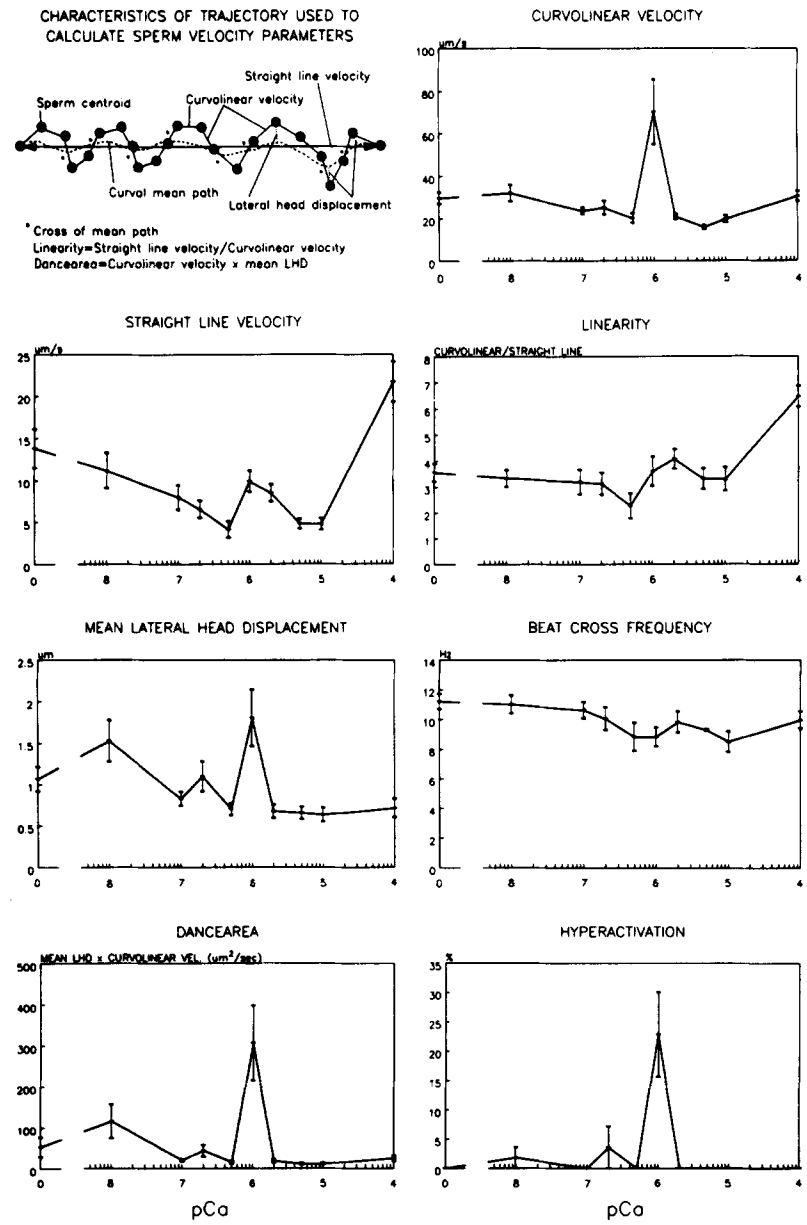

Figure 5. Effect of Ca^{2+} on motility of reactivated dog sperm. Dog sperm models were reactivated for 3 min with ATP and cAMP in the absence or presence of free Ca^{2+} ranging between 5×10^{-8} and 5×10^{-4} M. Motility parameters were determined by digital image processing. A description of the characteristics of sperm trajectory used to derive the velocity parameters is given in the upper left panel. Curvolinear velocity (*μm/s*) represents the sum of the distances traveled between subsequent 1/30 s centroid positions divided by the total sampling time. Straight line velocity is the straight line distance divided by time. Mean lateral head displacement (*μm*) represents 2 × the mean of the maxima of the perpendicular distance of the sperm head from the curval mean path. Beat cross frequency represents the rate (*Hz*) at which the sperm head crosses the mean path. A more detailed description of these parameters and their measurement has already been described by Mack et al., 1988. Hyperactivation is a type of trajectory characteristic of fertile spermatozoa displayed temporally during the acquisition of the ability to fertilize the egg. The method for determining hyperactivation is given by Robertson, L., D. P. Wolf, and J. S. Tash (manuscript submitted for publication). The results depicted represent ±SEM for 40–80 cells.

cAMP. As summarized in Fig. 5, Ca^{2+} showed discrete patterns of influence on different parameters of sperm motility over a narrow range of Ca^{2+} between 5×10^{-7} and 2×10^{-6} M. All parameters of motility except beat cross frequency were stimulated between 5×10^{-7} and 1×10^{-6} M Ca^{2+}. It is particularly interesting that the effect of Ca^{2+} was diminished sharply as Ca^{2+} increased above 1 μM. The low values for the parameters observed at 1 μM Ca^{2+} remained low for all parameters except straight line velocity and linearity.

Effect of Calmodulin-dependent Protein Phosphatase on Motility

The possibility that calmodulin-dependent protein phosphatase may serve a functional role in the control of sperm movement was examined. Sperm models were prepared using Hepes lysis buffer modified to contain 0.1 mM BAPTA instead of EGTA. Sperm (25 μl) were incubated for 2 min at 37°C in the presence of (*a*) no additions; or (*b*) Ca^{2+} (2 μM free); or (*c*) Ca^{2+} and purified calmodulin-dependent protein phosphatase (1.2 μM); or (*d*) Ca^{2+}, phosphatase, and 5 μM SK1. The incubations were terminated by addition of 25 μl Hepes buffer containing 10 mM BAPTA to reduce the free Ca^{2+} to 1.4 nM. Reactivation was then initiated by addition of ATP and cAMP. The results are summarized in Fig. 6. Relative to incubation without any additions, Ca^{2+} alone stimulated straight-line velocity, linearity, and beat cross frequency, but inhibited curvolinear velocity and lateral head displacement. When calcineurin was added, the effects of Ca^{2+} on all parameters except straight-line velocity were reversed. With curvolinear velocity and lateral head displacement, the phosphatase not only reversed the inhibitory effect of Ca^{2+} but resulted in a stimulation of these parameters relative to the control incubation. With beat cross frequency, a relative inhibition was induced by phosphatase treatment. SK1, in every case, reversed the effect noted by the phosphatase. The magnitude of the reversal by SK1 however was quite variable. In the case of curvolinear velocity, straight line velocity, and lateral head displacement, SK1 inhibited the parameters to levels below the controls. With linearity and beat cross frequency, SK1 resulted in partial restoration to levels produced by Ca^{2+} alone.

Discussion

Protein phosphatase activity has been demonstrated in *Paramecium* cilia (Lewis and Nelson, 1980, 1981) that may, at least in part, be represented by calmodulin-dependent protein phosphatase (Klumpp et al., 1983). While protein phosphatase activity has also been identified in spermatozoa from a variety of species including bovine (Tang and Hoskins, 1975), goat (Barua et al., 1985), and sea urchin (Swarup and Garbers, 1982), the extent of the contribution to this activity by calcineurin was not determined. Our results demonstrate the presence of calmodulin-dependent protein phosphatase (calcineurin) in dog sperm by four different criteria. These criteria were (*a*) immunoblot identification; (*b*) indirect immunofluorescent localization; (*c*) blockage of endogenous Ca^{2+}-dependent dephosphorylation with monospecific antibodies to bovine brain calcineurin; and (*d*) biochemical assay of activity that was Ca^{2+}-dependent and blocked by SK1 and antibodies against authentic calmodulin-dependent protein phosphatase.

A role for protein dephosphorylation in the regulation of sperm motility has already been suggested by previous studies from this laboratory. Tash et al. (1984) demonstrated that incubation of phosphorylated NP-40 sperm extracts with extracted sperm (as a source of protein phosphatase) inhibited the ability of the extract to subsequently reactivate sperm. The inhibition of reactivation was related to the amount of phosphate removed from the extract proteins. Takahashi et al. (1985) and Brokaw (1987) used partially purified muscle phosphoprotein phosphatase to dephosphorylate sperm cAMP-dependent phosphoproteins. Removal of phosphate markedly reduced the extent of reactivation upon addition of ATP. Rephosphorylation of the dephosphorylated sperm proteins reversed the reduction in reactivation produced by phosphatase treatment (Tash et al., 1984; Takahasi et al., 1985).

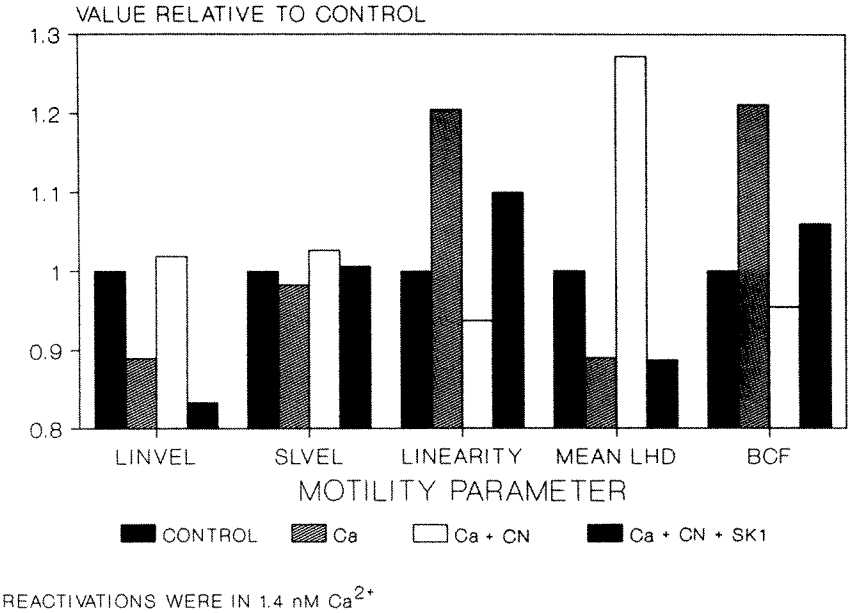

Figure 6. Effect of calmodulin-dependent phosphatase on motility. Permeabilized sperm were incubated for 2 min at 37°C in the presence of 2 μm free Ca^{2+} (*Ca*), Ca^{2+} plus 1.2 μM purified calmodulin-dependent protein phosphatase (*Ca + CN*), Ca^{2+} plus phosphatase plus SK1 (*Ca + CN + SK1*), or with no additions (*CONTROL*). Free Ca^{2+} was then lowered to 1.4 nM with 5.05 mM BAPTA and reactivation initiated with ATP and cAMP. Motility parameters were quantitated by digital image analysis. Results are expressed as a ratio relative to the value obtained for the control for each parameter.

The observation that the calmodulin-dependent protein phosphatase was present in isolated flagella and the dynein-containing high-salt extract from dog, pig, and sea urchin sperm suggests that this enzyme may play a role in the regulation of all types of flagellar movement. Preliminary results have demonstrated the cosedimentation of the phosphatase with dynein after resolution on sucrose gradients. Our observations represent the first demonstration of a biochemically defined calmodulin-dependent regulatory enzyme pathway that may be directly connected with dynein. The presence of this enzyme in the dynein fraction would be consistent with and could explain the observations of Brokaw and Nagayama (1985). They found that the ability of calmodulin to confer potential asymmetry upon sea urchin flagella was dependent upon the environment at the time of permeabilization with respect to the presence or absence of Ca^{2+}. If Ca^{2+} was present at the time of permeabilization then flagella were responsive to subsequent addition of calmodulin and displayed asymmetric bending patterns. If cells were lysed in the absence of Ca^{2+} then flagella did not display a change in waveform with subsequent addition of calmodulin and remained symmetric. When the presence of the calmodulin-dependent phosphatase is considered, it is possible that when cells were lysed in the presence of Ca^{2+} the phosphatase was activated due to the presence of Ca^{2+} and endogenous calmodulin. This would have created a net dephosphorylated state for a subset of sperm phosphoproteins. If any of these phosphoproteins were calmodulin-regulated enzymes which must be dephosphorylated for calmodulin to regulate them, then preexposure to Ca^{2+} would render these enzymes capable of being regulated by subsequent addition of calmodulin. Conversely, in the absence of Ca^{2+} the proteins would remain phosphorylated and be severely attenuated with respect to subsequent challenge with calmodulin.

Our results suggest that calmodulin may regulate motility via indirect macromolecular interactions determined by calmodulin-regulated enzymes such as the protein phosphatase. Our results could also explain the apparent discrepancy between results in *Tetrahymena* (Blum et al., 1980; Blum and Hayes, 1984*a*, *b*) in which calmodulin may regulate dynein and results in *Chlamydomonas* (Gitelman and Witman, 1980) that suggest no such interaction. If calmodulin regulates motility by an indirect effect on dynein through the phosphatase or through an effect on other axonemal components (such as dephosphorylation of radial spokes) then the conditions of axoneme preparation with respect to the Ca^{2+} environment may be critical to preserving such an interaction as suggested by Brokaw and Nagayama (1985). The apparent lack of a calmodulin effect in *Chlamydomonas* should be reexamined in this light. The present studies suggest that exogenous calmodulin-dependent protein phosphatase has a stimulatory effect on curvolinear velocity and lateral head amplitude, and an inhibitory effect on beat cross frequency. This conclusion is based on the fact that these parameters of motility were modulated in direct relation to the expected activity state of the added enzyme. The phosphatase-dependent alterations in velocity, lateral head amplitude (a manifestation of the magnitude of flagellar bending), and beat cross frequency are consistent with an effect of the enzyme on translation of dynein function to waveform production.

One question raised by these results is whether the endogenous phosphatase regulates the same parameters of motility observed in the enzyme addition experiments. The fact that some parameters of motility were stimulated by reactivation in the presence of Ca^{2+} (Fig. 5) but inhibited by Ca^{2} pretreatment without added phosphatase (Fig. 6), such as curvolinear velocity and mean lateral head displacement, suggests that Ca^{2+} exerts its influence on motility by affecting more than one component of the mechanochemical axonemal mechanism. Whether the other Ca^{2+}- and phosphatase-altered parameters of motility also comprise an involvement of the phosphatase or involve other Ca^{2+}-dependent processes remains to be determined. In relation to this question, the presence of other Ca^{2+}-dependent regulatory processes involved in the control of flagellar movement is supported by the observed bidirectional effect of Ca^{2+} on protein phosphorylation and the presence of multiple phosphoproteins affected by Ca^{2+}.

The fact that so many phosphoproteins were revealed by the inhibition of endogenous calmodulin-dependent protein phosphatase suggests that this enzyme represents a major factor determining the steady-state level of phosphorylation of sperm proteins. This observation is particularly interesting in view of the fact that, of all the protein phosphatases identified to date, calmodulin-dependent phosphatase represents the enzyme of narrowest substrate specificity (Cohen, 1982). These findings are consistent with the suggestion that in motile flagellar systems proteins are in a net phosphorylated state and that regulation may involve carefully coordinated dephosphorylation (Tash and Means, 1983). Further experimental evidence in support of this hypothesis is that stimulation of motility by egg factors in sea urchin sperm is coupled to the selective dephosphorylation of guanylyl cyclase (Ward et al., 1985; Bentley et al., 1986).

Several major questions are posed by the identification of multiple phosphoprotein substrates in sperm. The first question concerns the localization of the substrates for the phosphatase. Are the substrates within dynein or associated with other axonemal components, and what is the relation of axokinin to dynein? Our observations have identified 14 substrates for this phosphatase in whole sperm models, one of which is axokinin. Which of these proteins, if any, may be associated with dynein will certainly need to be examined. In this connection, Piperno and Luck (1982) have demonstrated that several heavy subunits and two 28-kD subunits and a 19-kD subunit of *Chlamydomonas* dynein are phosphoproteins. Of these proteins, peptide 3 in Fig. 4 has a similar size and charge to the 19-kD phosphorylated subunit of dynein reported by Piperno and Luck (1981). The second major question concerns the nature of the protein kinases that phosphorylate these proteins. Are the substrates in the dynein phosphorylated in a cAMP- or Ca^{2+}–calmodulin-dependent manner? Finally and most importantly, what is the function of these substrates with respect to regulation of the mechanochemical function of dynein and its intrinsic ATPase activity; and how are these functions altered by phosphorylation and dephosphorylation? Our results suggest a direct involvement with the control of flagellar bending and the rate of microtubule sliding.

In conclusion, the identification of dynein-associated, calmodulin-dependent protein phosphatase and Ca^{2+}-regulated phosphorylation sites on sperm proteins opens several new avenues for investigation into the role of cAMP- and Ca^{2+}-dependent protein phosphorylation in the regulation of

sperm flagellar motility. First, the function of the Ca^{2+}-dependent phosphorylation sites in relation to the stimulatory effect of cAMP and axokinin upon sperm motility should be elucidated. Second, the mechanism whereby calmodulin-dependent protein dephosphorylation modulates axonemal function will need to be studied in much greater detail. Finally, the factors that determine the balance between cAMP and Ca^{2+}-dependent phosphorylation and dephosphorylation need to be identified. To solve these problems, the protein substrates for the phosphatase that regulate flagellar movement will need to be identified, isolated, and characterized with respect to their interaction with dynein and other components of the axoneme.

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