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

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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 (Mr 15,000) and the calmodulin-binding A subunit with an Mr of 63,000. The sperm A subunit is slightly higher in Mr 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 curvilinear 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.

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Dog sperm for reactivation were prepared from ejaculates as above, sus-
pended in Hepes reactivation buffer (10 mM Hepes, 2 mM EGTA, 1 mM
dithiothreitol [DTT], 5 mM MgSO4, 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 con-
centration of 0.015% (vol/vol). In preincubation experiments, EGTA was
replaced with 0.1 mM BAPTA (1,2-bis(o-aminophenoxy)-ethane-N,N',N'-
ten-tetraacetic acid; Molecular Probes, Eugene, OR) to allow adequate
buffering of Ca2+ 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, CaCl2 (1 x 10-3 to 1 x
10-4 M free cation concentration). The required amount of Ca2+ to give the
desired free Ca2+ 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 curvilinear velocity, straight line velocity, linearity, mean lateral
head displacement, beat cross frequency, and mean radius of curvative of tra-
etories were measured in reactivated sperm using the CellSoft real time digi-
tal image processing system (Version 3.51c, CryoResources Ltd., New York,
NY). These parameters have been defined by Mack et al. (1988) and Robert-
son, L., D. P. Wolf, and J. S. Tash (manuscript submitted for publication).
A chamber depth of 32 ¼m was used. CellSoft setup parameters were as
follows: number of frames to analyze, >113; data acquisition rate, 30
frames/s; minimum sampling for motility and Brownian motion filter, 4
points; minimum sampling for velocity, 13 points; maximum velocity, 400
µm/s; minimum velocity, 10 µm/s. For lateral head displacement and beat
cross frequency the parameters were fixed: minimum number of points, 13;
minimum velocity, 100 µm/s; minimum linearity, 0. For circular
motion parameters the following values were set: minimum number of
points, 13; minimum velocity, 100 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
Ca2+ concentration. Statistics for each parameter were calculated from the
individual measurements for each motile cell (40-80 motile cells were mea-
sured for each point).

Protein Phosphorylation
Protein phosphorylation was carried out under the same conditions used for
reactivation except that ATP was replaced with [γ-32P]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-M 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 based on its ability to inhibit endogenous sperm calmod-
ulin-dependent phosphatase activity by biochemical determinations. Phos-
tidylation was then initiated by the addition of [γ-32P]ATP and cAMP
and/or Ca2+ 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-nitro-
phenylphosphate (p-NPP) as a substrate by a modification of the method of
Palien 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 CaCl2, 12 mM MgCl2, pH 8.0), 1 µl calmodulin (1 mg/ml),
10 µl of 100 mM p-NPP, and H2O to a total assay volume of 200 µl. To de-
terminate protein phosphatase activity in the absence of Ca2+ EGTA was in-
cluded in the assay mixture to a final concentration of 8.2 mM to reduce
free Ca2+ 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., Ober-
lin, OH) with a chart recorder. Rates were determined from the linear portion
of the tracings using e0 = 15,300.

High Resolution Two-dimensional PAGE
Protein phosphorylation/dephosphorylation was analyzed by high resoluti-
ion two-dimenional PAGE using the Health Products MegaDalt multiple
gel casting system based on the method of Anderson and Anderson
(1989a, b), as used previously in this laboratory (Tash et al., 1984, 1986).
The first dimension was disc IEF using pH 3.5-10 amphotiles (a 2:1 mixture
of LKB and Pharmacia wide-range amphotiles, 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. (1961),
then dried and autoradiographed using Kodak X-5 film and Quanta
III intensifying screens. Autoradiographic signals were quantitated by digi-
tal image processing by the method of Mii and others (1982).

Indirect Immunofluorescence
Indirect immunofluorescence of calmodulin-dependent protein phosphatase
in sperm was performed by a previously published procedure (Tash and
Mann, 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
guant rabbit IgG (Miles Laboratories Inc., Naperville, IL) at 1:50 dilu-
tion. 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 elec-
trophoresis to nitrocellulose sheets by a modification (Guerrero 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 mg/ml and [3H]protein A followed by
autoradiography.

Crude Dynine Preparation
Dynine 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 Ca2+. High-salt extracts from dog and pig sperm were prepared by
essentially the same method with the following modifications. Sperm were
washed in Ca2+-free Krebs’ ringer phosphate and then suspended in hypo-
tonic Hepes buffer (10 mM Hepes, 1 mM DTT, 2 mM EGTA, 5 mM
MgSO4, 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 car-
rried out by the same method described above for sea urchin.

Calcineurin and Calcineurin Antibodies
Calcineurin was purified to apparent homogeneity from bovine brain as de-
scribed 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. Calcineurin was not detected in the
purified enzyme and the basal activity measured in the absence of calmodu-
lin 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 evi-
dent after incubation for 1 h at 30°C (Klee, C. B., unpublished observation).

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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 preparations were not tested directly, however, calcineurin purified on an antibody-Sepharose column yielded undegraded antibody. By Western immunoblot, the antibody recognized only the two subunits of calcineurin in crude brain extracts (Blumenthal et al., 1986), was 1.3 mol phosphate cleaved/min per mg protein.

The only enzyme known to catalyze protein dephosphorylation in a Ca\(^{2+}\)-dependent manner is the calmodulin-dependent protein 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 Mr \(61,000\) (A subunit) and the light chain (B subunit) at Mr \(15,000\). A similar pattern was noted in dog sperm (Sp). The A subunit in sperm displayed a slightly higher Mr of 63,000. Additional analysis demonstrated calcium-dependent \([\text{\textsuperscript{45}}\text{Ca}^{2+}\text{]}}\) binding to a sperm protein at 63,000 by the method of Hubbard, M., M. Krinks, and C. B. Klee (manuscript in preparation) and \([\text{\textsuperscript{45}}\text{Ca}^{2+}\text{]}}\) binding to a 15-kD sperm peptide by the method of Maruyama et al. (1984). The same size subunits (63 and 15 kD) were noted for the calcineurin from 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**

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**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 (Mr, 61,000). B designates the migration of the B subunit of bovine brain calcineurin (Mr, 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\textsuperscript{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 \textsuperscript{32}P\textsubscript{ATP} in the presence and absence of cAMP and/or Ca\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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 $^{32}$PATP 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 axokin 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$^{2+}$. SKI inhibited the Ca$^{2+}$-dependent phosphatase activity in the extracts with an apparent $K_i$ of 1.3 $\mu$M. Since not all the activity was Ca$^{2+}$ dependent and was not inhibited by SKI, 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 SKI 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 $5 \times 10^{-8}$ to $5 \times 10^{-4}$ M free Ca$^{2+}$ on motility of dog sperm reactivated with ATP and Table II. Quantitative Analysis of the Effect of Anticalcineurin on Axokin Phosphorylation

| Phosphorylation conditions | 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 $\mu$g/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 $^{32}$PATP, in the absence and/or presence of $5 \times 10^{-7}$ M cAMP or $1 \times 10^{-4}$ 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 axokin 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 SKI. 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 SKI (Blumenthal et al., 1985; sequence = LKYYLMRRKKNFIAYSAANRF-KKI) 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 Ca²⁺ 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 Ca²⁺ 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. Curvilinear 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 curvilinear 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, Ca²⁺ showed discrete patterns of influence on different parameters of sperm motility over a narrow range of Ca²⁺ 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 Ca²⁺. It is particularly interesting that the effect of Ca²⁺ was diminished sharply as Ca²⁺ increased above 1 µM. The low values for the parameters observed at 1 µM Ca²⁺ 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) Ca²⁺ (2 µM free); or (c) Ca²⁺ and purified calmodulin-dependent protein phosphatase (1.2 µM); or (d) Ca²⁺, phosphatase, and 5 µM SKI. The incubations were terminated by addition of 25 µl Hepes buffer containing 10 mM BAPTA to reduce the free Ca²⁺ 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, Ca²⁺ alone stimulated straight-line velocity, linearity, and beat cross frequency, but inhibited curvilinear velocity and lateral head displacement. When calcineurin was added, the effects of Ca²⁺ on all parameters except straight-line velocity were reversed. With curvilinear velocity and lateral head displacement, the phosphatase not only reversed the inhibitory effect of Ca²⁺ 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. SKI, in every case, reversed the effect noted by the phosphatase. The magnitude of the reversal by SKI however was quite variable. In the case of curvilinear velocity, straight line velocity, and lateral head displacement, SKI inhibited the parameters to levels below the controls. With linearity and beat cross frequency, SKI resulted in partial restoration to levels produced by Ca²⁺ alone.

**Discussion**

Protein phosphatase activity has been demonstrated in *Paramaecium* 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 Ca²⁺-dependent dephosphorylation with monospecific antibodies to bovine brain calcineurin; and (d) biochemical assay of activity that was Ca²⁺-dependent and blocked by SKI 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 Ca²⁺-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; Takahashi 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 Ca²⁺ (*Ca*), Ca²⁺ plus 1.2 µM purified calmodulin-dependent protein phosphatase (*Ca + CN*), Ca²⁺ plus phosphatase plus SKI (*Ca + CN + SKI*), or with no additions (*CONTROL*). Free Ca²⁺ 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.](image-url)
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\textsuperscript{2+}. If Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} the phosphatase was activated due to the presence of Ca\textsuperscript{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\textsuperscript{2+} would render these enzymes capable of being regulated by subsequent addition of calmodulin. Conversely, in the absence of Ca\textsuperscript{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, 1984a, 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\textsuperscript{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 curvilinear 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\textsuperscript{2+} (Fig. 5) but inhibited by Ca\textsuperscript{2+} pretreatment without added phosphatase (Fig. 6), such as curvilinear velocity and mean lateral head displacement, suggests that Ca\textsuperscript{2+} exerts its influence on motility by affecting more than one component of the mechanochemical axonemal mechanism. Whether the other Ca\textsuperscript{2+}- and phosphatase-altered parameters of motility also comprise an involvement of the phosphatase or involve other Ca\textsuperscript{2+}-dependent processes remains to be determined. In relation to this question, the presence of other Ca\textsuperscript{2+}-dependent regulatory processes involved in the control of flagellar movement is supported by the observed bidirectional effect of Ca\textsuperscript{2+} on protein phosphorylation and the presence of multiple phosphoproteins affected by Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}-regulated phosphorylation sites on sperm proteins opens several new avenues for investigation into the role of cAMP- and Ca\textsuperscript{2+}-dependent protein phosphorylation in the regulation of
spem flagellar motility. First, the function of the Ca²⁺-dependent phosphorylation sites in relation to the stimulatory effect of cAMP and axokin 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 Ca²⁺-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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