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Regulation of Protein Phosphorylation and Motility of Sperm by Cyclic Adenosine Monophosphate and Calcium

J. S. TASH[1] and A. R. MEANS

Department of Cell Biology
Baylor College of Medicine
Houston, Texas 77030

ABSTRACT

Motility and protein phosphorylation have been measured under identical experimental conditions in ejaculated dog sperm lysed with low concentrations of Triton X-100 and reactivated with [γ-^{32}P] ATP. Cyclic AMP stimulates motility and protein phosphorylation while calcium inhibits motility and the overall incorporation of phosphate into endogenous proteins. Analysis of ^{32}P-labeled sperm proteins on 1- and 2-dimensional polyacrylamide gels demonstrates that an enhanced phosphorylation of a defined number of specific proteins is associated with cAMP-stimulated motility. A major axonemal protein, namely tubulin, has been tentatively identified as a phosphoprotein subject to regulation by cAMP. The phosphorylation of tubulin is almost completely dependent upon cAMP and is not affected by μM calcium. On the other hand, the cAMP-dependent stimulated phosphorylation of the other sperm proteins still occurs, but in most instances at a reduced rate in the presence of calcium. Two high molecular weight (M_r) phosphoproteins (350,000 and 260,000 daltons) whose phosphorylation states are modified by cAMP and calcium also were identified. It is suggested that 1 or both these proteins may be high M_r subunits of dynein. The phosphorylation of 1 of these proteins is stimulated by cAMP, but not affected by calcium; the other is stimulated by cAMP and inhibited by calcium. Three major cAMP-independent phosphoproteins of M_r 98,000, 43,000 and 26,000 have been identified. The phosphorylation of the 98,000 M_r protein is markedly reduced by micromolar calcium and not restored by cAMP. Using anticalmodulin drugs to inhibit motility, we suggest that the inhibitory effects of calcium on flagellar motility may be mediated in part by calmodulin. We conclude that the regulation of flagellar motility by cAMP and calcium includes mechanisms involving the control of the phosphorylation state of sperm proteins, some of which may be axonemal components.

INTRODUCTION

A correlation between changes in cAMP content and motility in sperm has been known for some time (for reviews see Garbers and Kopf, 1980; Mann and Lutwak-Mann, 1981). Cyclic AMP levels are positively correlated with increased motility, while experimental conditions which reversibly immobilize sperm, in turn, produce reversible reductions in cAMP content (Tash and Mann, 1973; Cascieri et al., 1976). More direct evidence that cAMP may actually regulate sperm motility was presented by Lindemann (1978) who showed that addition of cAMP to ATP-reactived detergent-lysed bovine sperm models produced measurable increases in beat frequency and the proportion of motile cells. The only known action of cAMP in eukaryotic cells is the stimulation of protein phosphorylation via activation of cAMP-dependent protein kinase (Rosen et al., 1975). Cyclic AMP-dependent protein kinase has been shown to be present in sperm in a variety of species (Garbers et al., 1973; Hoskins et al., 1972, 1974; Lee and Iverson, 1975) and represents a significant proportion of the protein present in sperm extracts. A number of protein substrates for cAMP-dependent protein kinase have been identified in both membrane and soluble fractions from bovine and human sperm homogenates (Hoskins and Stephens, 1975; Huacuja et al., 1977; Brandt and Hoskins, 1980). However, studies to date concerning the control of protein phosphorylation by cAMP in sperm have been limited to homogenates and subcellular fractions where structural integrity and motility have been destroyed.

In contrast to the positive regulatory effects of cAMP on motility, elevated calcium levels are correlated with inhibition of motility (Gibbons and Gibbons, 1980; Gibbons, 1980). In systems using detergent-lysed models and calcium/ EGTA buffers to control free calcium,

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[1] Correspondence.

a dose-dependent relationship between calcium and flagellar wave symmetry has been determined (Hyams and Borisy, 1978; Bessen et al., 1980). At very low concentrations of calcium (10^{-7} M or less) flagella beat symmetrically. As the calcium level is elevated, the wave form becomes more asymmetric until a concentration is reached (10^{-4} M) where motility is inhibited altogether. Brokaw et al. (1974) have shown that the effects of added calcium on sea urchin sperm flagellar motion occur when the cells are first lysed in the presence of calcium. Similar effects were observed independent of calcium if calcium was omitted from the extraction solution. Calcium has also been shown to be involved in the control of cell motility via mechanisms which may also involve in part the control of protein phosphorylation (Means et al., 1981).

We therefore examined the effects of cAMP and calcium on protein phosphorylation and motility of dog sperm. To this end we developed a detergent-lysed model which can be reactivated by ATP using very low concentrations of Triton X-100. Using [γ-^{32}P] ATP to reactivate the flagella and cAMP and/or calcium to modulate motility, both protein phosphorylation and motility could be measured under identical experimental conditions.

MATERIALS AND METHODS

Preparation of Spermatozoa

Dog semen (0.5 to 2 ml per ejaculate) was collected into 15 ml conical polycarbonate centrifuge tubes using a latex rubber sheath, and cooled slowly over a 4 h period to 4°C. The immobilized sperm were washed free of seminal plasma and cytoplasmic droplets by a modification of the low speed centrifugation method of Harrison (1971). The cold semen was diluted with 3 vol of cold calcium-free Krebs-Ringer phosphate solution (pH 7.4) (Dawson et al., 1974) and centrifuged at 400 X g for 15 min. The supernate was discarded and the wash procedure repeated 2 more times. Removal of cytoplasmic droplets was confirmed by phase microscopy. The pelleted sperm were resuspended to a concentration of 10^5 to 10^6 cells/μl in calcium-free Krebs-Ringer phosphate buffer if intact cells were required or in lysis buffer consisting of 10 mM N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES) containing 1 mM ethyleneglycol-bis (β-aminoethyl ether) N, N'-tetracetic acid (EGTA), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 mM NaCl and 0.01% (v/v) Triton X-100 (Sigma) to prepare models for reactivation.

Indirect Immunofluorescence

Washed intact sperm were attached to poly-L-lysine-coated 11 X 22 mm glass coverslips, washed with phosphate buffered saline (97.6 mM NaCl, 25 mM KCl, 8.3 mM glucose, 3.7 mM Na_2HPO_4 and 0.3 mM KH_2PO_4 at pH 7.3), then fixed in 3% formaldehyde followed by absolute acetone as described by Brinkley et al. (1980). The catalytic subunit of cAMP-dependent protein kinase was localized using 200 μg/ml affinity-purified rabbit antibody against glutaraldehyde-cross-linked catalytic subunit purified from bovine heart (Tash et al., 1981). The protein inhibitor of cAMP-dependent protein kinase was localized using 100 μg/ml affinity purified sheep antibody against the inhibitor from rat testis (Tash et al., 1980). Calmodulin was localized using 100 μg/ml affinity purified sheep antibody against calmodulin purified from rat testis (Dedman et al., 1978; Chafouleas et al., 1979). Tubulin was localized using 100 μg/ml affinity purified rabbit antibody against 6S tubulin purified from bovine brain (Brinkley et al., 1980). Control staining was performed using preimmune immunoglobulin G (IgG) (protein A-Sepharose affinity purified; Tash et al., 1980) or antibody preabsorbed with excess antigen. Fluorescein-tagged second antibody (1/40 dilutions) consisted of rabbit anti-sheep IgG and goat anti-rabbit IgG (Maloy Labs.).

Electron Microscopy

Intact, lysed or sonicated sperm were pelleted in a 1.5 ml conical centrifuge tube and fixed for 1.5 h in 3% glutaraldehyde in 200 mM sodium cacodylate at pH 7.2. The pellets were post-fixed in 1% OsO_4 for 1 h; then dehydrated stepwise in ethanol and embedded in Spurr's medium. Thin sections (700 Å) were cut and stained with lead and uranyl acetate; then viewed and photographed with a Seimens 102 electron microscope.

Motility Measurements

Sperm motility (intact cells or reactivated models) was recorded on videotape using a Dage 650 video camera connected to a Panasonic WV-5310 monitor and Panasonic time lapse videotape recorder (NV-8030). The percent of motile cells was determined in at least 20 fields (40 X objective and 10 X eyepiece) containing 5 to 20 cells per field. Test groups consisted of at least 100 motile cells. Motility measurements were made at 1–2 min after reactivation.

Assay of cAMP-Dependent Protein Kinase

Cyclic AMP-dependent protein kinase was assayed in lysed and sonicated sperm using exogenous histone (Sigma type IIB) as substrate. Samples were assayed in duplicate in the presence and absence of 1 μM cAMP and 0.3 mM [γ-^{32}P] ATP (200–1000 cpm/pmole) by the procedure of Fakunding and Means (1977). Activity was expressed as an activity ratio, which is the activity measured in the absence of cAMP divided by that determined in the presence of cAMP, and by specific activity (per mg protein).

Endogenous Phospborylation

Lysed sperm were reactivated by the addition of 10 μl of lysed sperm suspension to 50 μl of lysis buffer containing [γ-^{32}P] ATP (8,000–10,000 cpm/pmole) at a final concentration of 1.0 mM. Cyclic AMP, when tested, was present at a final concentration of 1 to 10

$\times 10^{-6}$ M. Calcium, when tested, was present at a final free calcium concentration of 1×10^{-7} to 1×10^{-4} M. Free calcium was calculated from the EGTA/calcium buffer program described by Potter and Gergely (1975). Reactions (total vol of 60 μl) in duplicate were allowed to proceed for 0 to 60 min at 30°C, and terminated by the addition of 2.0 ml of 10% (w/v) trichloroacetic acid (TCA) and 10 μl of bovine serum albumin at 10 mg/ml. After 30 min at 0°C the suspensions were centrifuged for 10 min at 1500 × g, the supernate was discarded and the pellet dissolved in 50 μl of 1 M NaOH. Fresh 10% TCA was added to reprecipitate the proteins and the wash procedure repeated a total of 4 times. The final washed pellet was dissolved in NCS (Amersham): water (9:1) and radioactivity was determined by liquid scintillation spectrometry. Background radioactivity was determined by using heat-inactivated sperm suspensions or by the addition of TCA immediately prior to the addition of the sperm suspension.

Polyacrylamide Gel Electrophoresis and Autoradiography

^{32}P-labeled phosphoproteins were analyzed by both 1- and 2-dimensional polyacrylamide gel electrophoresis. Phosphorylation reactions to be analyzed in 1 dimension only were terminated by the addition of 20 μl of concentrated (4-fold) Laemmli (1970) sample buffer and heated at 90°C fo 10 min, while reactions to be analyzed in 2 dimensions were terminated by the addition of 72 mg solid urea (BioRad electrophoresis grade) and 11 μl of water. Prior to electrophoresis all samples were homogenized for 8 min with a Bronwill Biosonic IV sonicator and centrifuged for 2 h at 100,000 × g to remove DNA. One-dimensional electrophoresis was performed using SDS-containing 6–20% polyacrylamide gradient slab gels (Laemmli, 1970). Two-dimensional electrophoresis was carried out by the procedure of O'Farrell (1975). The first dimension was isoelectrofocusing using pH 3.5 to 10 ampholines (LKB); the second dimension was SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using a 6–20% gradient gel. After electrophoresis, the dye front (Pyronin Y) containing [γ-^{32}P]ATP and $^{32}P_i$ was sliced from the gel, and the remainder of the gel was fixed overnight in methanol:acetic acid:water (40:7:53), stained 2 h at 30°C with 0.05% Coomassie Brilliant Blue G in methanol:acetic acid:water; then destained with 7% acetic acid. The gel was then equilibrated with 7% acetic acid containing 2% glycerol, dried and exposed to Cronex (Dupont) x-ray film. After development the film was scanned with a Helena Quick Scan Jr.

Immunoprecipitation

Immunoprecipitation of tubulin was carried out by the procedure of Chafouleas et al. (1981) using monospecific antibodies to tubulin prepared and kindly supplied by Brinkley et al. (1980). Blank values were obtained by the simultaneous addition of sperm suspension and reaction termination buffer to the reaction mixture. Reaction termination buffer contained 10 mM sodium phosphate, 150 mM NaCl, 1 mM EGTA, 5 mM ATP, 1% Triton X-100 and 1% deoxycholate, pH 7.5. Samples were sonicated for 8 min, then centrifuged at 2,000 × g for 2 min to obtain an extract for use in immunoprecipitation.

[γ-^{32}P]ATP

[γ-^{32}P]ATP was synthesized by the procedure of Schendel and Wells (1973).

Protein Determination

Protein was measured by the procedure of Bradford (1976) using bovine gamma globulin as standard.

Materials

Carrier free [^{32}P]orthophosphate was purchased from New England Nuclear. Compounds W12 [N-(4-aminobutyl)-2-naphthalene sulfonamide] and W13 [N-(4-aminobutyl)-5-chloro-2-napththalene sulfonamide] were obtained from Prof. H. Hidaka, Mie University, Japan. ATP (vanadate free), cAMP, Triton X-100 and EGTA were purchased from Sigma. All reagents used were of analytical quality.

RESULTS

Localization of Catalytic Subunit of cAMP-Dependent Protein Kinase, Its Protein Inhibitor, Calmodulin and Tubulin in Sperm

The catalytic subunit of cAMP-dependent protein kinase, its protein inhibitor, calmodulin and tubulin were localized in washed dog sperm by indirect immunofluorescence using affinity purified monospecific antibodies to each of these proteins. The catalytic subunit was localized in 3 areas of the sperm (Fig. 1A): *1*) in the head, as a band in the post-acrosomal region, possibly the equatorial ring; *2*) at the base of the axoneme in the region of the centriole; and *3*) in the principal piece of the tail behind the midpiece. No staining in these regions was observed when preimmune IgG or preabsorbed antibody was tested. Occasionally the acrosomal region of sperm heads was stained; however, the proportion of cells showing this type of staining was identical to that for dead cells as assessed by stain exclusion (as per Dott and Foster, 1972). The protein inhibitor of cAMP-dependent protein kinase was localized in 2 areas of the sperm (Fig. 1B): *1*) at the base of the axoneme in the region of the centriole; and *2*) in the principal piece of the tail behind the midpiece. Controls using preimmune IgG or preabsorbed antibody failed to show fluorescence in these areas. Occasionally, staining was observed in the head, but this was not preabsorbable with excess antigen. Calmodulin was shown to be present; *1*) throughout the tail, including a bright spot

associated with the region of the centriole, and 2) in a band in the post-acrosomal region, presumably the equatorial ring (Fig. 1C). Controls using preabsorbed antibody failed to show any staining pattern in sperm. Tubulin (Fig. 1D) was localized throughout the tail and as a bright spot at the base of the axoneme, presumably in the region of the centriole. Preimmune IgG and preabsorbed antibody controls showed complete absence of staining in the cells.

In the course of these experiments it was noted that cytoplasmic droplets showed very bright staining for both calmodulin and the catalytic subunit of cAMP-dependent protein kinase (data not shown). It is for this reason that ejaculated rather than epididymal sperm were used in all experiments. In addition, the washed sperm were routinely checked for removal of cytoplasmic droplets.

Effect of Calmodulin Drugs on Sperm Motility

The question of whether the effects of calcium in sperm are mediated by calmodulin was investigated by determining the effect of the anticalmodulin drug, W13, and its inactive analogue, W12, on motility of intact (nonlysed) dog sperm (Fig. 2). These 2 naphthalenesulfonamide derivatives bind to calmodulin in a calcium-dependent manner, however, the dechlorinated compound (W12) has a 5-fold lower affinity for displacement of [^{3}H]W-7 (Hidaka et al., 1980) from calmodulin as well as for the inhibition of Ca^{++}-calmodulin activation of phosphodiesterase and myosin light chain kinase activity (Chafouleas et al., 1982). While both compounds differ in their anticalmodulin activity, they possess similar hydrophobicity indices and both easily traverse the cell membrane (Chafouleas et al., 1981). Both drugs

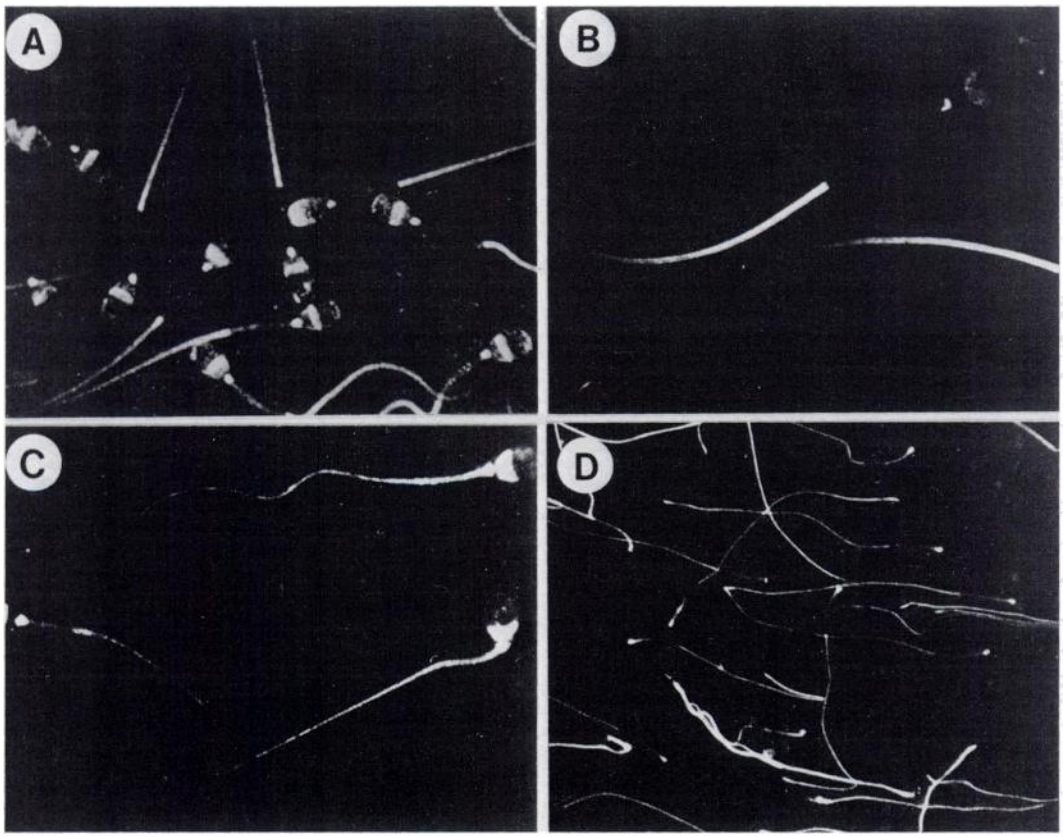

FIG. 1. Indirect immunofluorescent localization of catalytic subunit of cAMP-dependent protein kinase, protein kinase inhibitor, calmodulin and tubulin in ejaculated dog sperm. Ejaculated dog sperm were washed to remove seminal plasma and cytoplasmic droplets, then processed for indirect immunofluorescence microscopy as described in *Materials and Methods*. *A*) catalytic subunit of cAMP-dependent protein kinase (×1098); *B*) protein kinase inhibitor (×2745); *C*) calmodulin (×1372); and *D*) tubulin (×1098).

were tested in the range between 1×10^{-5} and 1×10^{-3} M. Neither W12 nor W13 had any effect on motility at concentrations below 4×10^{-5} M. As W13 was increased from 4×10^{-5} to 4×10^{-4} M, there was a marked increase in the proportion of cells showing abnormal swimming patterns, i.e. curled tails and/or circular swimming patterns; both alterations in motility were coincident. At concentrations of W13 at 5×10^{-3} M and above, motility was inhibited completely; tails remained curled but beating stopped. On the other hand, W12, the inactive (dechlorinated) analogue had little effect upon the proportion of motile cells even at 1×10^{-3} M. Furthermore at 4×10^{-4} M W12, only 6% of the cells showed abnormal motility while the equivalent concentration of W13 produced abnormal motility in all cells.

Development of the Lysed Sperm Model

The optimal concentration of Triton X-100 needed to prepare flagella which could be reactivated by ATP was established by a number of criteria, including permeability to stain, ability to assay cAMP-dependent protein kinase using exogenous substrates and the ability to restore motility by the addition of ATP. Figure 3 shows the effect of increasing detergent concentration on the ability of sperm to exclude stain. Sperm were stained using eosin/nigrosin "live/dead" stain (Dott and Foster, 1972) after exposure for 1 min to various concentrations of Triton X-100 in lysis buffer (see *Materials and Methods*). At detergent concentrations of 0.004% (approximately 60 μM) or below, cells showed 30–40% staining. This is within the normal range for sperm (Dott

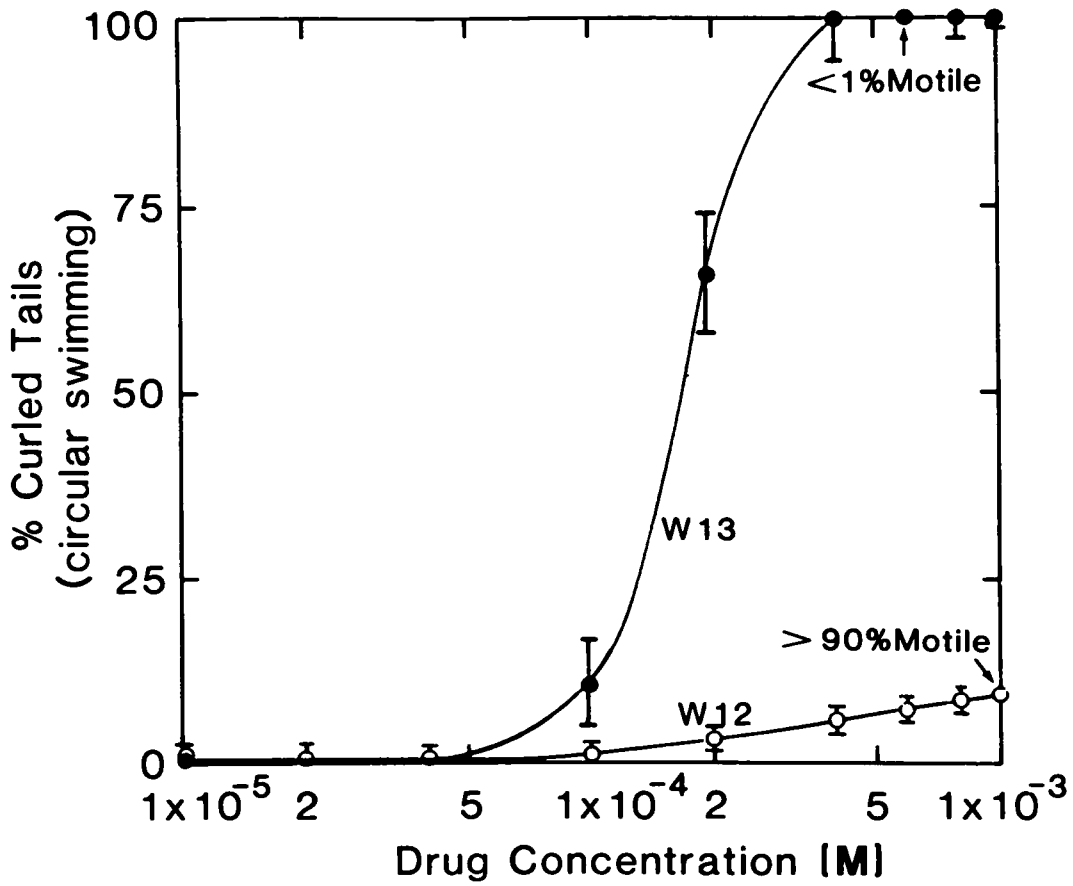

FIG. 2. Effect of calmodulin drugs on sperm motility. Washed dog sperm were monitored for motility in calcium-free Krebs-Ringer phosphate buffer containing 1×10^{-5} to 1×10^{-3} M W12 or W13 as described in *Materials and Methods*. Cells were incubated with the drugs for 1 min at 30°C prior to measurement of motility using a video tape recorder connected to a phase-contrast microscope.

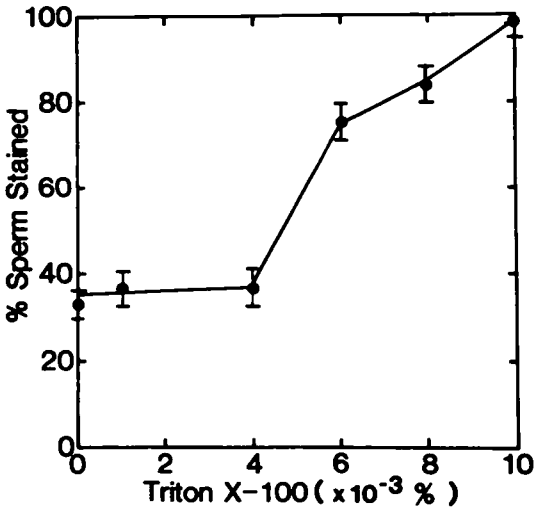

FIG. 3. Effect of Triton X-100 on stain exclusion of washed dog sperm. Washed dog sperm were incubated for 1 min in calcium-free Krebs-Ringer phosphate solution (see *Materials and Methods*) containing 0 to 0.01% (v/v) Triton X-100, then stained with eosin/nigrosin "live/dead" stain (Dott and Foster, 1972). Only cells that showed complete exclusion of stain were scored as unstained.

and Foster, 1972). At higher detergent concentrations, there was a dose-dependent increase in the proportion of stained cells. The minimal concentration of Triton X-100 that produced 100% stained cells was 0.01% (approximately 160 μM). This is also the critical micelle concentration for Triton X-100 (Rohm and Haas technical data for Triton detergents).

The second criterion, the ability to assay cAMP-dependent protein kinase in the lysed cells, was established by measuring enzyme activity using exogenous substrates after cells were treated with increasing concentrations of Triton X-100. The activity measured in the lysed cells was compared with the activity that could be measured using cell homogenates. Homogenates were prepared by sonicating sperm suspensions in lysis buffer without detergent for 0 to 2 min (30 sec intervals with 30 sec cooling periods in an ice cooling bath using a Bronwill Bionosonik IV at maximum setting). Efficacy in the assay of cAMP-dependent protein kinase would be manifested by an increase in specific activity of the enzyme paralleled by an increased dependence upon cAMP for activity (i.e., decreased activity ratio). Figure 4 summarizes the results of these experiments. Optimal cAMP-dependent protein kinase activity was demonstrable in homogenized sperm after only 30 sec of sonication (Fig. 4A). Nearly identical cAMP-dependent protein kinase activity was achieved when sperm were lysed with 0.01% Triton X-100 (Fig. 4B). The further increases in specific activity of cAMP-dependent protein kinase noted with higher concentrations of detergent probably result from detegent-induced conformational changes in the histone and not Triton X-100-induced activation of enzyme activity as had been reported by Sugden et al. (1976). It should be noted that in cells not treated with detergent or sonication, some protein kinase activity was detectable. However, none of this activity was cAMP-dependent.

The final criterion was evaluated by reactivating with increasing concentrations of ATP sperm that were lysed with 0.01% Triton X-100 (Table 1). As the concentration of ATP was increased, the proportion of motile cells increased, reaching a peak at between 200 and 500 μM ATP with a somewhat lower plateau between 1 and 2 mM ATP. Since the normal concentration of ATP in sperm is around 1 mM (Brooks, 1970), the effects of cAMP and calcium on motility were determined using 1 mM ATP. Addition of cAMP (10 μM) produced a 2.5-fold increase in the proportion of motile cells. In addition, the wave amplitude was increased in all cells while little change in the beat frequency was observed after addition of cAMP. Beat frequencies of 10–15 Hz were obtained in the reactivated models. The stimulatory effect of cAMP on motility was observed even at the lower concentration of ATP (200 μM) where higher basal motility was observed. In additional experiments, the stimulatory effects of cAMP on motility could be prevented if the sperm were reactivated in the presence of 50 ng of a purified preparation of the heat stable inhibitor of cAMP-dependent protein kinase (PKI). On the other hand, PKI had no effect on the basal motility observed in the absence of added cAMP. The stimulatory effects of cAMP on motility are contrasted by the inhibitory effects of calcium. In both the presence and absence of cAMP, nearly complete inhibition of motility was produced by 9 μM free calcium. In the presence of cAMP, half maximal inhibition of motility was achieved with 1.2 μM free calcium. As 9 μM calcium produced near complete inhibition of motility, subsequent experiments on the effect of calcium used this level of the cation.

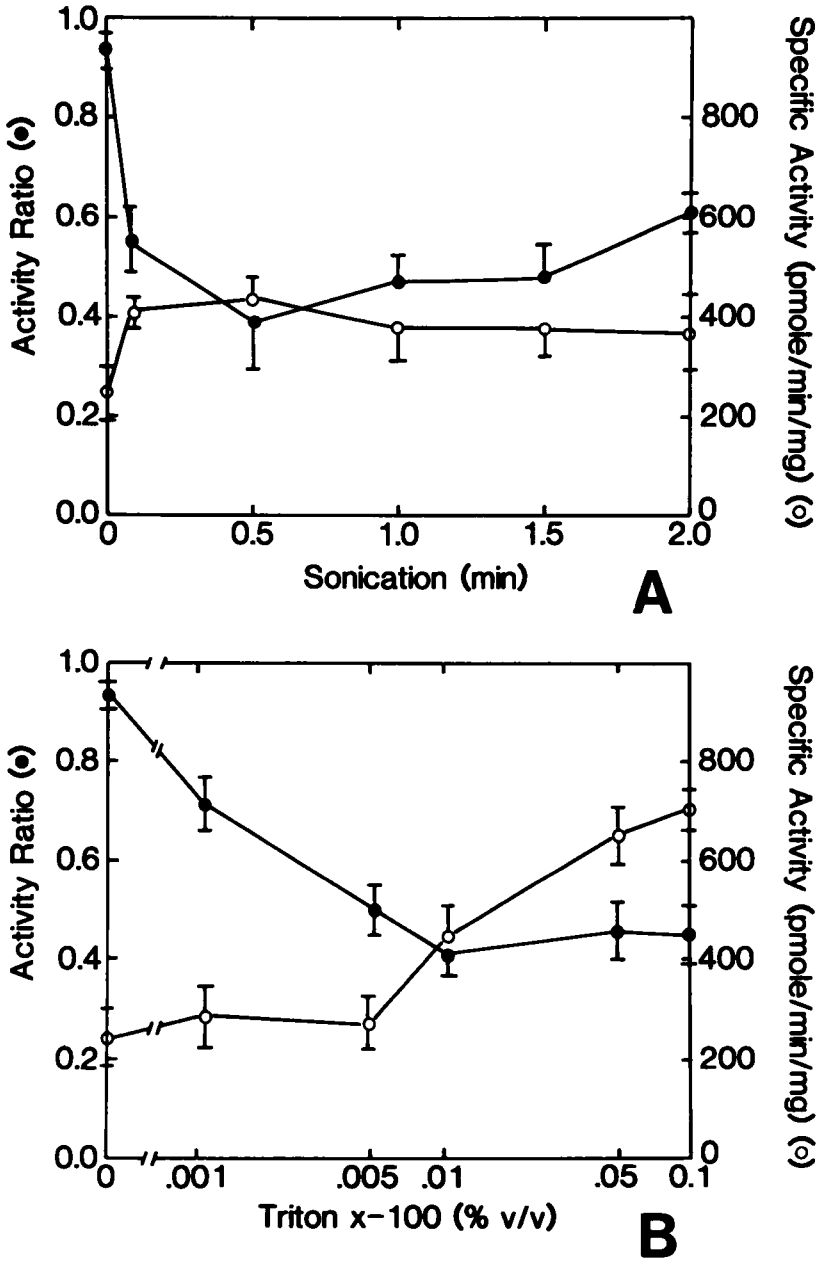

FIG. 4. Assay of cAMP-dependent protein kinase in Triton-lysed vs. sonicated sperm using exogenous substrates. Washed dog sperm were either: *A*) sonicated for 0 to 2 min (in a cooling bath) or *B*) lysed for 1 min with 0 to 0.1% Triton X-100; then assayed for cAMP-dependent protein kinase activity using exogenous cAMP, [γ-^{32}P] ATP and histone (Sigma Type II) as substrates (see *Materials and Methods*). Activity ratio is determined by measuring kinase activity in the presence and absence of 1 μM cAMP and calculated by dividing the activity measured in the absence of cAMP by that determined in the presence of cAMP; the greater the dependence on cAMP for activity, the lower the activity ratio. Specific activity was determined in the presence of cAMP.

TABLE 1. Effect of nucleotides and calcium on motility of reactivated sperm.[a]

ATP (μM)	cAMP	Ca^{++}	% Motile	% Change
0	–	–	0	
50	–	–	3.9	
100	–	–	9.8	
200	–	–	25.4	
500	–	–	20.7	
1000	–	–	13.3	
2000	–	–	13.1	
200	10 μM	–	32.3 (P<0.001)	+27%
1000	10 μM	–	32.3 (P<0.001)	+240%
1000	–	9 μM	0.9 (P<0.001)	−99%
1000	10 μM	9 μM	1.1 (P<0.001)	−99%
100	10 μM	1.2 μM	16.1 (P<0.001)	−50%

[a]Washed dog sperm were lysed with 0.01% Triton X-100 and reactivated with 0 to 200 μM ATP in the presence or absence of cAMP and/or calcium as indicated. Motility was determined as described in *Materials and Methods*. P values were determined with respect to cells reactivated with the equivalent concentration of ATP in the absence of both cAMP and calcium. The percentage of change in motility was calculated by dividing the value for motility in the presence of cAMP by that measured in the absence of cAMP at equivalent ATP concentrations.

Ultrastructure of Sperm Model

The ability not only to reactivate the lysed sperm by the addition of ATP, but also to modulate motility by the addition of cAMP and calcium suggested that the structural integrity of the cells had been preserved. This was confirmed by ultrastructural examination of the model. Figure 5A shows the ultrastructure of control sperm that had not been lysed or sonicated. The *inset* shows a cross section through the midpiece of the tail. Figure 5B shows the ultrastructure of a sperm that had been lysed with 0.01% Triton X-100. The membranes are still present and the overall structure appears to have remained intact. High magnification pictures of the detergent-treated sperm (not shown) revealed numerous pores in the membranes. A cross section through the tail (*inset*) shows that the detergent treatment has not affected the conformational array of the structural components of the axoneme. By comparison, Fig. 5C shows the ultrastructure of sperm that were sonicated for 30 sec. The *inset* shows what remains of the tails after such treatment.

Time-Dependent Endogenous Phosphorylation

Using [γ-^{32}P]ATP to reactivate the sperm, endogenous phosphorylation was monitored during a 60 min incubation period in the presence and absence of cAMP (Fig. 6). In the absence of cAMP (*closed circles*), there is a rapid initial rate of incorporation which falls to a somewhat slower rate after 10 min. In the presence of cAMP (*open circles*), the initial rate of phosporylation is 30 to 40% faster than in the absence of cAMP. After 10 min, however, the rate of phosphorylation that occurs in both the presence or absence of cAMP appears similar. This occurs even in the presence of 8-bromo-cAMP; thus, the loss in cAMP-stimulated activity is not due to hydrolysis of cAMP.

pH-Dependence of Endogenous Phosphorylation

The effect of cAMP on phosphorylation is most evident during the first few minutes of reaction. The effect of pH on endogenous phosphorylation during a 1 min incubation was examined (Fig. 7). In both the presence (*open circles*) or absence (*closed circles*) of cAMP, phosphorylation was relatively unaltered by changes in pH between 6.5 and 7.5. At pH 8.0, however, there was a slight drop in phosphorylation, both in the presence and absence of cAMP. It should be noted that while protein phosphorylation at the 2 extremes of pH tested were similar, at pH 6.5 cells were completely immotile while at pH 7.5 and 8.0, cells showed good motility (50–60% motile).

Effect of Fluoride on Endogenous Phosphorylation

Fluoride ions have been used as an inhibitor of protein phosphatases and ATPases in experiments to study phosphoproteins in sperm cytosol preparations (Brandt and Hoskins, 1980). The necessity of fluoride for the inhibition of protein phosphatases and ATPases in the reactivated sperm was tested during a 1 min reactivation period in the presence and absence of cAMP using sodium fluoride, ranging in concentration from 0 to 100 mM. Fluoride inhibited phosphorylation in a linear dose-dependent manner in both the presence and absence of cAMP. At 100 mM fluoride phosphorylation was 58% of that observed in the absence of fluoride, both in the presence and absence of cAMP, respectively. At all fluoride concentrations tested, the ratio of cAMP-dependent to cAMP-independent phosphorylation was constant at approximately 1.4.

Regulation of Phosphorylation by cAMP and Calcium

The regulation of protein phosphorylation in sperm by cAMP and calcium was examined in greater detail (Table 2). As observed earlier, cAMP stimulated protein phosphorylation by 30 to 40%. On the other hand, micromolar free calcium inhibited protein phosphorylation by almost 40%. The inhibition of phosphorylation by calcium is not due to depletion of ATP by calcium-dependent ATPases, since thin-layer chromatography of the reaction mixtures demonstrated no additional breakdown of ATP due to the presence of calcium (data not shown). The inhibitory effects of calcium on protein phosphorylation could be overcome by the added presence of cAMP; under these conditions protein phosphorylation was restored to the level observed in control cells that had been reactivated in the absence of both cAMP and calcium. It should be noted that even though phosphorylation is restored by cAMP in the presence of calcium, the cells remain immotile (Table 1). Whether the effects of cAMP on protein phosphorylation were in fact due to cAMP-dependent protein kinase was examined by determining the effect of added protein kinase inhibitor (PKI) on phosphorylation in the absence and presence of cAMP. PKI is a specific inhibitor of the catalytic subunit of cAMP-dependent protein kinase, and as such can be used to inhibit any phosphorylation catalyzed by a free catalytic subunit (Ashby and Walsh, 1973). The increased phosphorylation produced by cAMP was almost completely blocked by the addition of pure PKI. In the absence of cAMP, PKI had little effect upon the rate of protein phosphorylation. This is an important result since it demonstrates that the protein phosphorylation that occurs in the absence of cAMP (see Fig 6 for example) is catalyzed by a cAMP-independent protein kinase or kinases.

Analysis of Sperm Phosphoproteins

The experiments to determine the optimal lysis conditions for the sperm (Fig. 4) indicated that these cells contain protein kinase activity that is detectable in presumably intact cells (not sonicated or detergent-treated). This protein kinase activity could be attributed either to damaged cells or to kinase activity on the external surface of the sperm. To examine these possibilities, washed intact (nonlysed) sperm suspended in calcium free Krebs-Ringer phosphate (see *Materials and Methods*) were incubated for 1 min at 30°C in the presence of 1 mM [γ-^{32}P] ATP. The possible presence of endogenous sperm phosphoproteins was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 8 (*second lane*), no sperm phosphoproteins were detectable. In sharp contrast, in an identical number of sperm lysed with 0.01% Triton X-100, numerous phosphoproteins were detectable (Fig. 8, *third* through *sixth lanes*). These results suggest that very few of the sperm have been damaged to expose intracellular phosphorylation reactions. In addition, the protein kinase that is capable of phosphorylating exogenous substrates (Fig. 4) either does not phosphorylate proteins in or on sperm or the proteins are already phosphorylated and thus not able to accept ^{32}P.

The rapid but short-lived cAMP-dependent phosphorylation that occurs in the reactivated detergent-treated sperm suggests the presence of a small number of highly cAMP-dependent phosphoproteins. This suggestion was investigated by reactivating sperm for 1 min in the presence and absence of cAMP using [γ-^{32}P] ATP and subjecting the phosphorylated sperm to analysis by SDS-polyacrylamide gel electrophoresis. Figure 8 depicts the results of these experiments. In the absence of both cAMP and calcium, 3 major phosphoproteins of

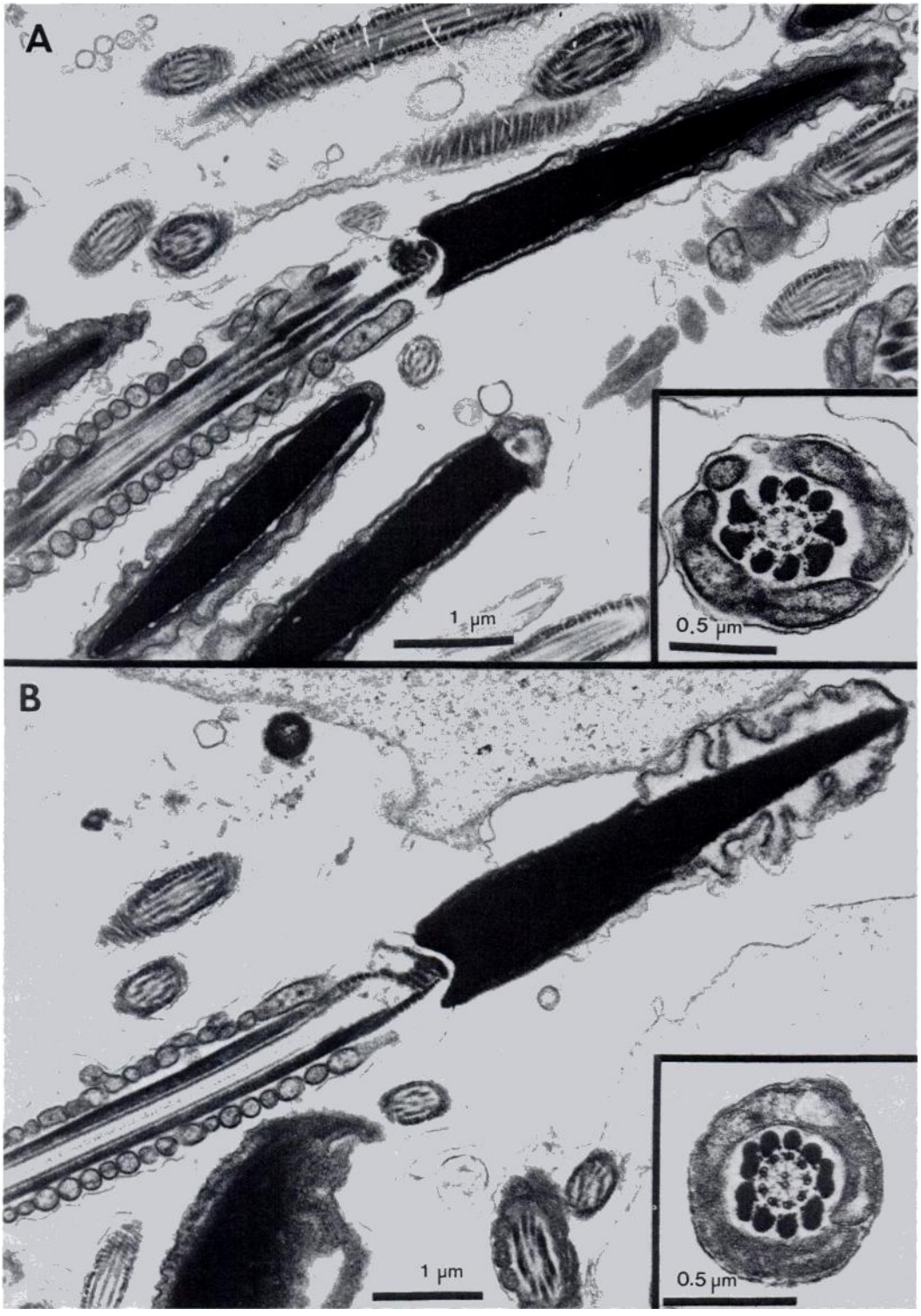

FIG. 5A, B.

M_r 98,000, 43,000 and 26,000 are evident (Fig. 8, *third lane*) with other minor phosphoproteins ranging between 350,000 and 21,400 M_r. Stimulation of motility with cAMP (Fig. 8, *fourth lane*) is accompanied by the enhanced phosphorylation of proteins of M_r 260,000, 220,000, 77,000, 68,000, 55,000, 49,000, 39,000, and 18,400, respectively (indicated by *arrows*). Several other phosphoproteins (M_r 350,000, 164,000, 122,000 and 21,400) also appear to be stimulated by cAMP. However, the level of these proteins makes the precise effect more difficult to assess with confidence. Of these phosphoproteins, the 68,000, 55,000, 39,000 and 18,400 M_r proteins are most prominent in their dependence upon cAMP for phosphorylation.

The inhibitory effect of calcium on protein phosphorylation was also examined by gel electrophoresis (Fig. 8, *fifth lane*). The most pronounced effect on protein phosphorylation produced by calcium (9 μM) in the absence of cAMP is the diminished phosphorylation of the 98,000 M_r protein. On the other hand, the other cAMP-independent phosphoproteins continued to be phosphorylated in the presence of calcium. As noted earlier, addition of cAMP to the reactivation medium in the presence of calcium restored the overall level of protein phosphorylation to that observed in cells reactivated in the absence of both cAMP and calcium (Table 2). However, analysis of such an experiment by gel electrophoresis (Fig. 8, *sixth lane*) demonstrated that the restoration of phosphorylation is due to the cAMP-stimulated phosphorylation of the cAMP-dependent phosphoproteins noted earlier (Fig. 8, *fourth lane*) and not to restored phosphorylation of calcium-inhibited phosphoproteins. The 98,000 M_r cAMP-independent phosphoproteins that

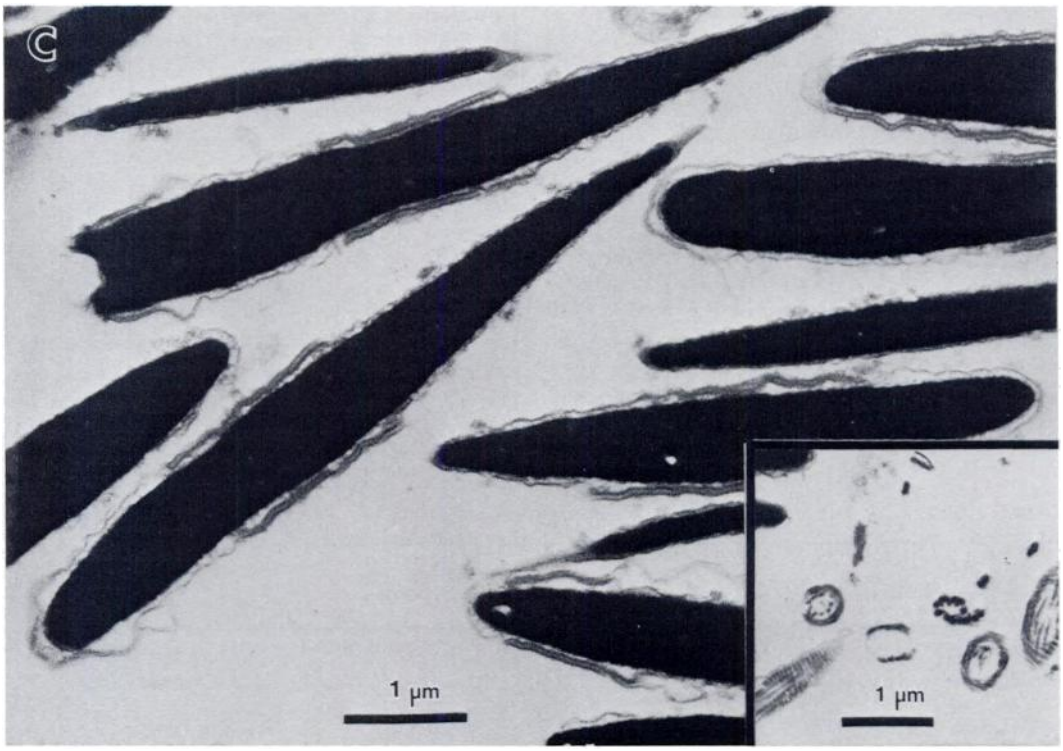

FIG. 5. Ultrastructure of control, Triton-lysed and sonicated dog sperm. Control (washed only) sperm and sperm either lysed with 0.01% Triton X-100 or sonicated for 30 sec were processed for electron microscopy by standard methods (see Materials and Methods). *A*) depicts a portion of a control washed dog sperm; the *inset* shows a cross section through the midpiece of a flagellum. *B*) shows a sperm lysed with 0.01% Triton X-100; the inset represents a cross section through the midpiece of a lysed sperm flagellum. *C*) shows what remains in a suspension of sperm sonicated for 30 sec; the *inset* shows typical highly fragmented tail pieces.

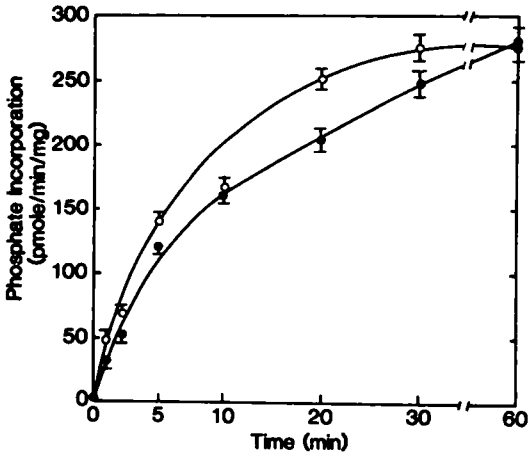

FIG. 6. Time-dependent phosphorylation of endogenous proteins in sperm reactivated in the presence and absence of cAMP. Dog sperm lysed with 0.01% Triton X-100 were reactivated with 1 mM [γ-^{32}P]ATP in the absence and presence of 10 μM cAMP. Phosphorylation of endogenous proteins was monitored by TCA precipitation as described in *Materials and Methods*. *Open circles* – phosphorylation in the presence of cAMP; *closed circles* – phosphorylation in the absence of cAMP.

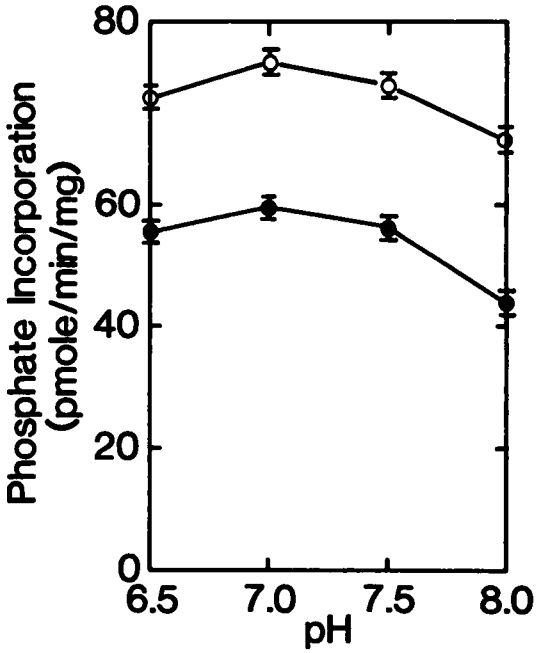

FIG. 7. pH-dependent phosphorylation of endogenous proteins in the presence and absence of cAMP. Lysed dog sperm were assayed over a 1 min period for endogenous phosphorylation in the presence (*open circles*) and absence (*closed circles*) of 10 μM cAMP. The reactivation buffer used to phosphorylate the sperm (see *Materials and Methods*) was modified to contain 10 mM HEPES as well as 10 mM PIPES (piperazine-N,N'-bis[2-ethane sulfonic acid) and 10 mM Tris to ensure buffering over the entire pH range tested.

were inhibited by calcium (Fig. 8, *fifth lane*), remained at a reduced level of phosphorylation in the presence of cAMP. Furthermore, in many cases, the cAMP-stimulated phosphorylation, although still pronounced, was at a somewhat reduced level of stimulation in the added presence of calcium (see Table 3). The cAMP-dependent phosphorylation of the 55,000 and 39,000 M_r proteins did not appear to be affected by calcium. For comparison, the *first lane* shows the M_r standards and the *last lane* in Fig. 8 shows the Coomassie blue-stained protein pattern for these samples. A list of the sperm phosphoproteins identified in these experiments

TABLE 2. Effect of cAMP and calcium on endogenous phosphorylation.[a]

Treatment	cAMP	Calcium	Phosphorylation (% of control)
Control	0	0	100
+cAMP/–Ca	2 μM	0	133.1 ± 2.1
–cAMP/+Ca	0	9 μM	62.7 ± 3.7
+cAMP/+Ca	2 μM	9 μM	100.1 ± 3.5
–cAMP/+PKI	0	0	104.1 ± 2.1
+cAMP+PKI	2 μM	0	106.8 ± 1.2

[a]Lysed sperm were reactivated with [γ-^{32}P]ATP for 1 min and phosphorylation of endogenous proteins determined as described in *Materials and Methods*. Protein kinase inhibitor (PKI), when added, was present in 50-fold molar excess to the maximal expected concentration of endogenous cAMP-dependent protein kinase activity.

and a summary of their regulation by cAMP and calcium is presented in Table 3.

Confirmation that the 55,000 M_r Phosphoprotein is Tubulin

The identity of the 55,000 M_r protein with tubulin was examined by 2 separate criteria. Sperm were reactivated with [γ-^{32}P] ATP in the presence and absence of cAMP and the reactions terminated with a buffer containing 1% Triton X-100 and 1% deoxycholate followed by sonication to solubilize the cells (see *Materials and Methods*). Aliquots were then immunopre-

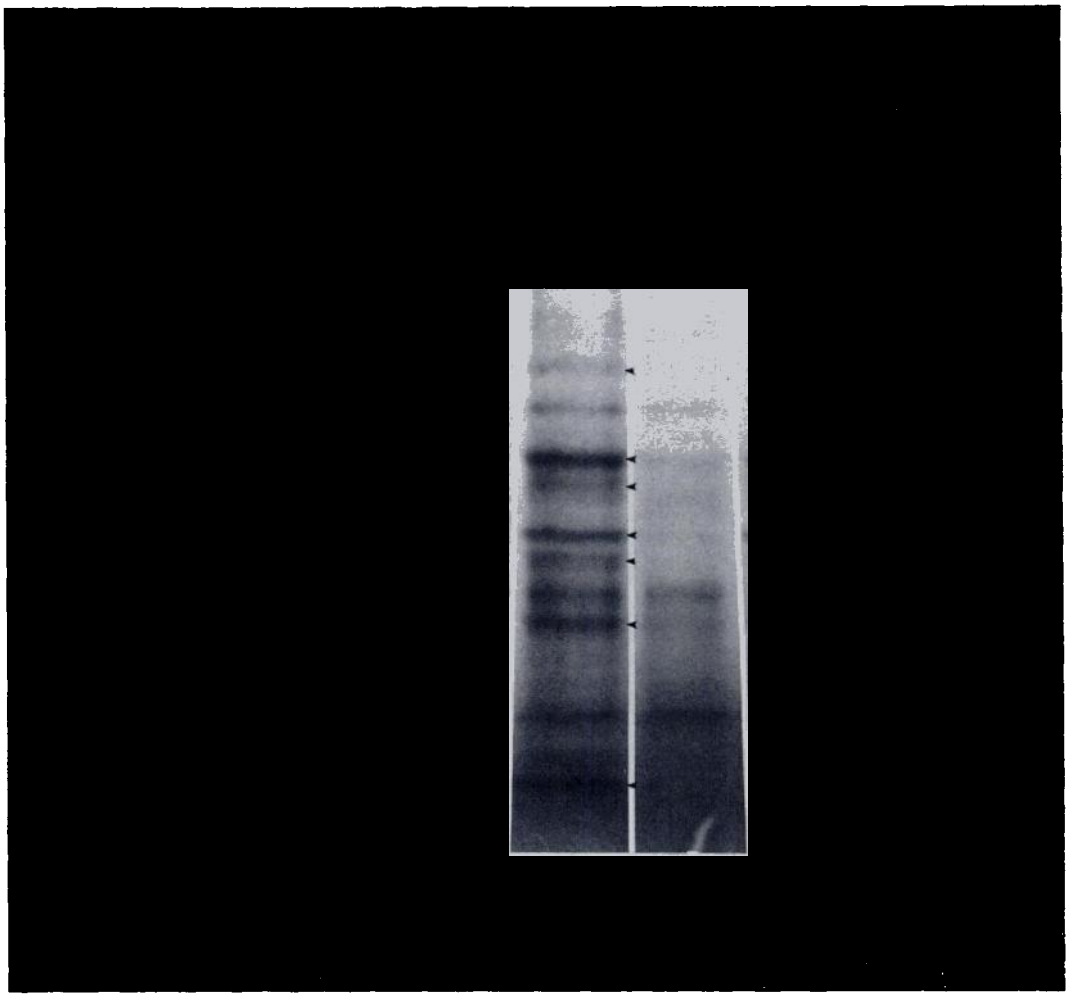

FIG. 8. SDS-polyacrylamide gradient gel analysis of sperm proteins phosphorylated in the presence or absence of cAMP and/or calcium. Detergent-treated sperm (or intact sperm as controls) were phosphorylated for 1 min in the absence or presence of 10 μM cAMP in the added presence or absence of 9 μM free calcium. The phosphorylated sperm were then subjected to analysis on 6–20% polyacrylamide gradient slab gels containing SDS (see *Materials and Methods*) and autoradiography. Each slot represents the same number of sperm and identical exposure to the x-ray film and subsequent photographic exposures. The *first lane* represents standard molecular weight markers with relative molecular weights (M_r) indicated. The *second lane* represents intact control sperm (not detergent-treated) incubated in calcium-free Krebs-Ringer phosphate for 1 min with [γ-^{32}P] ATP. The *third lane* represents phosphoproteins in detergent-treated sperm in the absence of both cAMP and calcium. The *fourth lane* represents sperm phosphoproteins present in the presence of cAMP and the absence of calcium. The *arrows* indicate the most prominent phosphoproteins stimulated by cAMP. The *fifth lane* represents phosphoproteins in the absence of cAMP, but in the presence of 9 μM free calcium. The *sixth lane* represents phosphoproteins in the presence of both cAMP and calcium. The *seventh lane* depicts the Coomassie blue-stained sperm protein pattern for these lanes.

TABLE 3. Phosphoproteins detected in [γ-^{32}P] ATP-reactivated dog sperm.[a]

Phosphoprotein M_r	cAMP Effect	Ca Effect
350,000	+	NE
→ 260,000	+	Inhibition of cAMP effect
→ 220,000	+	Inhibition of cAMP effect
190,000	NE	NE
164,000	+	NE
→ 122,000	+	Inhibition of cAMP effect
98,000	NE	Inhibition both ± cAMP
→ 77,000	+11.6-fold	40% Inhibition of cAMP effect
→ 68,000	+	Inhibition of cAMP effect
→ 55,000	+17.3-fold	NE
→ 49,000	+	Inhibition of cAMP effect
43,000	NE	NE
→ 39,000	+4.1-fold	NE
26,000	NE	NE
21,400	+	NE
→ 18,400	+7.8-fold	50% Inhibition of cAMP effect

[a]Detergent-treated dog sperm were reactivated for 1 min with [γ-^{32}P] ATP. Phosphoproteins were analyzed on 6–20% SDS-polyacrylamide slab gels and autoradiography (see Fig. 8). The relative molecular weights (M_r) of the 350,000, 260,000 and 220,000 M_r proteins are approximate since these values were not bracketed by standard M_r markers. Prominent cAMP-dependent proteins which are marked by *arrows* are also indicated by *arrows* in Fig. 8. All values for phosphorylation effects were calculated from densitometric scans of the autoradiographs taken at the same gain and zero settings. Where an effect by cAMP or calcium is noted without a value, the signal was too low to accurately calculate values. NE – little or no effect noted.

cipitated with monospecific antibodies to tubulin or total ^{32}P incorporation was determined by TCA precipitation. In this experiment (Table 4), overall phosphorylation was stimulated 46%. In the absence of cAMP about 2% of the total incorporated radioactivity was recoverable in the tubulin immunoprecipitate. After activation in the presence of cAMP, there was a 9-fold increase in the radioactivity recovered in the tubulin immunoprecipitate. Furthermore, phosphotubulin accounted for as much as 12% of the total sperm phosphoprotein in the presence of cAMP.

Further verification that tubulin is a sperm cAMP-dependent phosphoprotein was obtained by analyzing the sperm phosphoproteins on 2-dimensional polyacrylamide gels (see *Materials and Methods*). A portion of such a gel showing the region where tubulin migrates is shown in Fig. 9. Figure 9A shows the Coomassie blue-stained protein pattern. Both α and β tubulin are indicated. The *open arrow* indicates the position where the 55,000 M_r sperm phosphoprotein migrates, autoradiograms of which are depicted in 9B (without cAMP) and 9C (with cAMP), respectively. The phosphoprotein comigrates with the acid portion of the spot corresponding to the α-subunit of tubulin.

TABLE 4. Immunoprecipitation of sperm [^{32}P] phosphoprotein with antitubulin.[a]

	Total incorporation (cpm)	Antitubulin immunoppt. (cpm)	% of Total
–cAMP	3079	60	1.9%
+cAMP	4482 (46%)	540 (900%)	12.0%

[a]Lysed sperm were reactivated for 1 min with [γ-^{32}P] ATP in the presence or absence of cAMP. Aliquots were assayed for total ^{32}P incorporation by TCA precipitation or for ^{32}P incorporation into tubulin by immunoprecipitation with monospecific antibodies to tubulin as described in *Materials and Methods*.

DISCUSSION

With the use of a low concentration of Triton X-100, a lysed sperm model was developed which allowed motility and protein phosphorylation to be measured under identical experimental conditions. Motility of the reactivated model could be stimulated with micromolar levels of cAMP and inhibited with micromolar levels of free calcium. Under these conditions, overall phosphorylation of endogenous proteins was stimulated by cAMP and inhibited by calcium. The stimulation of protein phosphorylation by cAMP is consistent with previous observations concerning the presence of cAMP-dependent protein kinase in sperm (Garbers et al., 1973; Hoskins et al., 1972, 1974; Lee and Iverson, 1976). With the use of SDS-polyacrylamide gel electrophoresis and autoradiography we have been able to identify specific protein substrates for both cAMP-dependent and cAMP-independent protein kinases within sperm. The major cAMP-dependent proteins appear to be almost completely dependent upon cAMP for phosphorylation. This is born out by the near complete inhibition of cAMP-stimulated phosphorylation by added protein kinase inhibitor (PKI) and by the fact that the inhibitor did not further reduce the level of phosphorylation measured in the absence of cAMP. These results were paralleled by the ability of PKI to block the cAMP effects on motility. The observation that added PKI did not reduce the level of phosphorylation that occured in the absence of cAMP (Table 2) suggests that the phosphorylation that occurs in the absence of cAMP can be attributed entirely to cAMP-independent protein kinases.

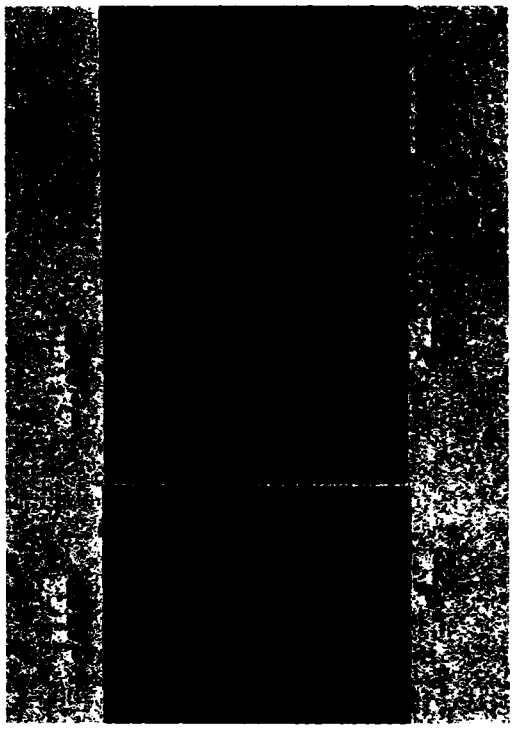

FIG. 9. Two-dimensional polyacrylamide gel analysis of sperm phosphoproteins in the region where tubulin migrates. Lysed sperm were reactivated for 1 min with [^{32}P]ATP in the presence and absence of cAMP, then subjected to 2-dimensional polyacrylamide gel electrophoresis. This figure shows the region of the gel where tubulin migrates. *A*) shows the Coomassie blue-stained protein pattern; the α- and β-subunits of tubulin are marked, respectively. The *open arrow* indicates the position of the gel corresponding to the location of the ^{32}P-labeled 55,000 M_r protein. *B*) autoradiogram of the same region of the gel showing phosphoproteins present in the absence of cAMP. The positions where α- and β- tubulin migrate are indicated, respectively. *C*) autoradiogram of the same region of the gel showing phosphorylation obtained in the presence of cAMP. Autoradiograms were obtained from identical numbers of cells, and were exposed to film for the same period of time.

The fact that motility of the reactivated sperm can occur in the absence of added cAMP suggests that the cAMP-dependent phosphorylation is not entirely necessary for the production of motility, but rather is involved in the modulation of motility. It should be noted, however, that when the molar concentration of cAMP is calculated based on cAMP content per sperm (see Garbers and Kopf, 1980) and water content per sperm (Drevius, 1972), then it appears that cAMP levels in normal freshly isolated cells are sufficiently high to fully activate the cAMP-dependent protein kinase contained within. cAMP has 2 major effects on motility. On one hand, it stimulates the proportion of cells that are motile; on the other, it appears to modify the flagellar wave form to produce increased swimming velocity. In these experiments, cAMP modified the waveform by increasing the wave amplitude. No significant changes in beat frequency were noted. Lindemann (1978) has shown that cAMP will also increase the proportion of bovine sperm which can be reactivated. Bovine sperm were found to increase beat frequency in response to cAMP; whether wave amplitude was affected was not mentioned. In either case, an increase in wave amplitude or beat frequency would facilitate an

increase in forward velocity. It is evident from our results concerning the effect of cAMP on protein phosphorylation that a number of proteins may be involved in translating the phosphorylation changes into alterations in motility. The question of why cAMP would increase the proportion of motile cells also needs to be approached. The use of isolated sperm flagella and axonemes will help to answer these questions.

The inhibitory effects of calcium on flagellar motility were accompanied by an inhibition of the phosphorylation of a 98,000 M_r cAMP-independent phosphoprotein and a reduction in the cAMP-stimulated phosphorylation of some proteins. Sperm are known to contain Ca^{++}-stimulated ATPase activity (Voglmayr et al., 1969; Forrester and Bradley, 1980), which might account for the diminution of phosphorylation in the presence of calcium due to substrate depletion. However, thin-layer chromatographic analysis of the phosphorylation reaction mixtures failed to show additional hydrolysis of ATP in the presence of calcium. The inhibition of protein phosphorylation produced by calcium could thus be explained by *1*) specific inhibition of protein kinases, *2*) calcium-dependent activation of a phosphoprotein phosphatase, or *3*) calcium induced conformational changes within the sperm which prevent substrate-kinase interactions. Evidence for the first 2 possibilities is lacking; however calcium is known to inhibit dynein arm detachment during microtubule sliding (Zanetti et al., 1979). Whether this relates directly to the inhibition of protein phosphorylation remains to be determined. All 3 possibilities require further examination. Results reported by Hyne and Garbers (1979a,b) have suggested an involvement of calcium in the regulation of cAMP formation in guinea pig sperm. It is unlikely that calcium is stimulating the production of significant levels of cAMP in the experiments reported here, since calcium alone did not enhance phosphorylation of the proteins found to be stimulated by cAMP.

Our observation by indirect immunofluorescence that the catalytic subunit of cAMP-dependent protein kinase is localized in both the head and tail of the sperm suggest that all of the cAMP-dependent phosphorylation that occurs within the sperm may be directly related to cAMP effects of flagellar motility. One cAMP-dependent substrate that we have identified which can be directly related to motility is tubulin. The identity of the 55,000 M_r phosphoprotein with tubulin, in particular the α-subunit, was determined by immunoprecipitation and by 2-dimensional gel electrophoresis.

There is conflicting evidence in the literature concerning whether or not flagellar tubulin is a substrate for cAMP-dependent protein kinase. Brandt and Hoskins (1980) identified a 55,000 dalton cAMP-dependent protein in extracts from sonicated bovine epididymal sperm. The 55,000 M_r protein did not bind [^{3}H] colchicine. It must be noted that colchicine will bind only to free tubulin dimers (M_r of 110,000). Furthermore, whether sonication under the conditions used would in fact depolymerize the tubulin to give tubulin dimers or a mixture containing nonsedimentable microtubule fragments, denatured tubulin monomers and some dimers, was not determined. There is substantial evidence to support our finding that tubulin is a phosphoprotein (Lagnado et al., 1972; Eipper 1972, 1974; Piras and Piras, 1974; Sandoval and Cuatrecasas, 1976; Ikeda and Steiner, 1979; Coughlin et al., 1980).

With regard to the cAMP-dependence of tubulin phosphorylation, it should be noted that Ponstingl et al. (1981) have determined the complete amino acid sequence of α-tubulin from porcine brain. Of particular note, the amino acid at residue 340 shows microheterogeneity in that both threonine and serine can be identified in this position. The nucleotide sequence of the mRNA for α-tubulin is also known (Valenzuela et al., 1981) and the corresponding nucleotide sequence for the amino acid at position 340 could, by a single base change, account for the microheterogeneity. When serine is in this position, then the flanking n-terminal amino acid sequence (lys-arg-ser) defines a typical cAMP-dependent phosphorylation site (Kemp et al., 1977; Yeaman et al., 1977). These observations, when considered in light of the fact that the tubulins are represented by unlinked and dispersed gene families (Cleveland *et al.*, 1981) and that there are testis-specific tubulins (Kemphues et al., 1979), may help to account for the conflicting reports concerning whether or not tubulin is a cAMP-dependent phosphoprotein. The possibility that flagellar axonemes contain tubulin with a serine in position 340 or a similar phosphorylatable sequence within the molecule should be considered.

Dynein is another motility-related protein which may be subject to regulation by phos-

phorylation. A number of phosphoproteins were identified in these studies that had similar mobilities to the high M_r subunits of dyneins, ranging between approximately 260,000 and 350,000 daltons (Piperno and Luck, 1979; Gibbons et al., 1976). The phosphoproteins in this molecular weight range fall into 2 categories with regard to protein phosphorylation. The phosphorylation of the 260,000 M_r protein is stimulated by cAMP. However, this stimulation is blocked by the presence of calcium. The 350,000 M_r protein appears to be stimulated by cAMP, but unaffected by calcium. Whether these phosphoproteins are in fact dyneins remains to be determined. In this connection, Piperno and Luck (1981) have demonstrated that a high molecular weight subunit of inner arm dynein from *Chlamydomonas* flagella is a phosphoprotein. Furthermore, the possible regulation of dynein ATPase activity by phosphorylation reactions certainly merits investigation. In preliminary experiments, we have identified calmodulin binding proteins in the region of the gel where these phosphoproteins migrate. Ciliary dynein has been shown to be regulated by calmodulin by Blum et al. (1980).

Our results using the anticalmodulin drugs W13 and W12 suggest that the effects of micromolar calcium on flagellar motility may be mediated by calmodulin. Whether all the calcium effects on sperm motility are calmodulin-dependent remains to be determined. While there is calmodulin in the tail of sperm, most of this protein has been localized in the head portion of this cell type (Fig. 1, this paper; Jones et al., 1978, 1980; Feinberg et al., 1981). Calmodulin has been postulated to confer the calcium sensitivity to dynein ATPase (Blum et al., 1980). In terms of a role for calmodulin in the calcium-dependent regulation of protein phosphorylation in sperm we have found an enzyme in sperm extracts capable of phosphorylating exogenous myosin light chains (Guerriero, Tash and Means, unpublished observations). Whether sperm in fact contain a calmodulin-activated protein kinase similar to myosin light chain kinase remains to be determined. Further experimentation with the anticalmodulin drugs using reactivated sperm and isolated flagella is currently in progress.

Our results have demonstrated a parallel relationship between the regulation of flagellar motility by calcium and cAMP and the regulation of protein phosphorylation. These initial experiments were performed on whole sperm permeabilized with low levels of detergent to yield a model activated by ATP which was used for measuring both motility and protein phosphorylation under identical experimental conditions. The phosphorylation state of a defined number of specific proteins has been correlated with the effects of cAMP and calcium on motility. One phosphoprotein, namely tubulin, is clearly related to the control of flagellar motility. Another phosphoprotein that has been identified may well prove to be dynein. Current endeavors are being devoted to identifying and determining the localization of all the sperm phosphoproteins in relation to the flagellar axoneme and to characterize in greater detail the precise biochemical and mechanochemical mechanisms which relate the control by cAMP and calcium of protein phosphorylation to those of flagellar motility. In conclusion, the control of flagellar motility appears to be defined in part by mechanisms which include the regulation of protein phosphorylation.

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