Axokinin Phosphorylation by cAMP-dependent Protein Kinase Is Sufficient for Activation of Sperm Flagellar Motility

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Abstract. Using a selective inhibitor of cAMP-dependent protein kinase, *N*-[2(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8), the requirement for cAMP-dependent phosphoproteins in the initiation of dog sperm flagellar motility was examined. H-8 inhibited motility of live as well as reactivated sperm in a dose-dependent manner. The half-maximal inhibition of reactivated motility (32 μM) paralleled the inhibition of pure catalytic subunit of cAMP-dependent protein kinase (50 μM) measured under the same conditions. H-8 inhibited protein phosphorylation both in whole models and in isolated Nonidet P-40 (NP-40) extracts of sperm. Axokinin, the heat-stable NP-40–soluble protein whose phosphorylation is required for flagellar reactivation, represented 97% of the *de novo* phosphate incorporation in the NP-40 extract after stimulation by cAMP. 500 μM H-8 inhibited axokinin phosphorylation by 87%. When sperm were reactivated in the presence of up to 5 mM H-8 with NP-40 extract that had been prephosphorylated with cAMP-dependent protein kinase, then neither cAMP nor cAMP-dependent protein kinase activity was required for full flagellar reactivation. If sperm were rendered completely immotile by pretreatment with H-8, then the resulting model remained immotile in the continued presence of H-8 unless prephosphorylated axokinin was added. These results suggest that phosphorylated axokinin is not only required for flagellar reactivation but is sufficient as well.

PREVIOUS studies have demonstrated that stimulation of sperm motility by cAMP is mediated by phosphorylations catalyzed by cAMP-dependent protein kinase (reviewed by Tash and Means [16]). More recently, axokinin, a heat-stable, Nonidet P-40 (NP-40)[1]-soluble protein has been identified. Phosphorylation of axokinin by cAMP-dependent protein kinase is required for motility in reactivated sperm models. Axokinin has been shown to be identical in human, dog, and sea urchin sperm as well as in canine retina and trachea as judged by heat stability, size, charge, color after silver staining, phosphorylation, and cAMP-dependent stimulation of motility (17). Axokinin may be the factor identified in NP-40 extracts of starfish sperm that confers cAMP-dependence on motility of sea urchin sperm (9) and the cAMP-dependent motility factor identified in epididymal bovine sperm by Brandt and Hoskins (3).

A consistent observation in reactivated sperm of both invertebrates and vertebrates has been the significant proportion of motility that occurs upon reactivation with ATP alone (4, 9, 11, 13, 15, 17, 20). The ATP-dependent motility of dog and sea urchin sperm could be blocked by the heat-stable protein inhibitor of cAMP-dependent protein kinase (9, 17). These results suggest that most if not all of the motility that occurs in sperm requires the maintenance of cAMP-dependent protein phosphorylation. However, it was not known whether or not axokinin phosphorylation alone might be sufficient for full reactivation of flagellar motility.

An isoquinolinesulfonamide derivative, *N*-[2(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8), has recently been synthesized by Hidaka et al. (7). This compound is a selective inhibitor of cyclic nucleotide-dependent protein kinases and is competitive with respect to ATP. Advantages of this compound over the peptide inhibitor of cAMP-dependent protein kinase are its ability to cross the plasma membrane and its selective inhibition of both cAMP- and cGMP-dependent protein kinases (7).

Using H-8 in live dog sperm and in reactivated models, we have examined the extent to which cAMP-dependent phosphorylation of sperm proteins, and specifically axokinin, is required for sperm flagellar motility. Evidence presented here suggests that not only is axokinin phosphorylation by cAMP-dependent protein kinase required for flagellar motility but it is sufficient as well.

Materials and Methods

Preparation of Sperm

Dog sperm were prepared free from seminal plasma and cytoplasmic droplets by slow cooling and washing at 600 *g* in calcium-free Krebs-Ringer's phosphate buffer containing 250 mg/ml fructose as described previously (15).

1. *Abbreviations used in this paper*: H-8, *N*-[2(methylamino)ethyl]-5-isoquinolinesulfonamide; NP-40, Nonidet P-40.

Flagellar Reactivation and Motility Analysis

Sperm for reactivation were prepared at 4°C as described by Tash et al. (17) using 0.015% NP-40 (BDH Chemicals Ltd., Poole, England) in 10 mM Hepes buffer containing 5 mM $MgSO_4$, 1 mM 2-mercaptoethanol, 2 mM EGTA, and 100 mM NaCl. Motility was reactivated by the addition of 100 μM ATP and 5×10^{-7} M cAMP and warming to 30°C. Where tested H-8 was added to a final concentration between 1×10^{-8} and 5×10^{-3} M. Motility was analyzed by digital image processing using the CellSoft (version 2.0) image analysis system (Cryo Resources Ltd., New York, NY). Data were collected at 30 video frames per second. 15 raster scans were collected (representing every other video field over a duration of 0.5 s), and at least 10 adjacent points per sperm trajectory were used in velocity and linearity analyses. Data were collected between the first and second minute of reactivation until at least 300 cells had been analyzed (usually three to five 0.5-s collections). A more detailed description of the CellSoft digital imaging technique for sperm motility analysis has been presented by Tash and Means (18). All experiments were performed in at least duplicate samples. Results are presented as means of the replicates. Reactivated models stick to the microscope slide by the head (even in the presence of 3% bovine serum albumin) making velocity determinations impossible. Results on models are thus restricted to percent motility, where a cell is counted as motile if the flagellum displayed readily visible and regular beating.

Preparation of NP-40 Extracts

Non-heat-treated NP-40 extracts of dog sperm were prepared as described by Tash et al. (17). Heat-treated phosphorylated NP-40 extracts were prepared in one of two ways: (*a*) NP-40 extract was phosphorylated with ATP and cAMP as described below, then heat-treated at 100°C for 5 min, or (*b*) extract was heat-treated, then phosphorylated with ATP and catalytic subunit of cAMP-dependent protein kinase, and then heat-treated again. In both cases, the extracts after heat-treatment were centrifuged at 10,000 *g*, again at 14,000 *g* (2°C), then dialyzed against Hepes reactivation buffer to remove nucleotides.

Protein Phosphorylation

Protein phosphorylation was carried out for 2 min under reactivation conditions by substituting for non-labeled ATP, [γ-^{32}P]ATP at a specific activity of ~1,000 cpm/pmol. Phosphorylation of isolated NP-40 extract was performed in reactivation buffer (see above) using either non-labeled or [^{32}P]ATP (17) and 5×10^{-7} cAMP. NP-40 extracts phosphorylated after heat treatment were prepared as above with the addition of 5 μg pure catalytic subunit of cAMP-dependent protein kinase (19) instead of cAMP. All phosphorylations were carried out for 2 min since this is the time during which cAMP produced the maximal rate of increase in motility as compared with cells reactivated without cAMP (15).

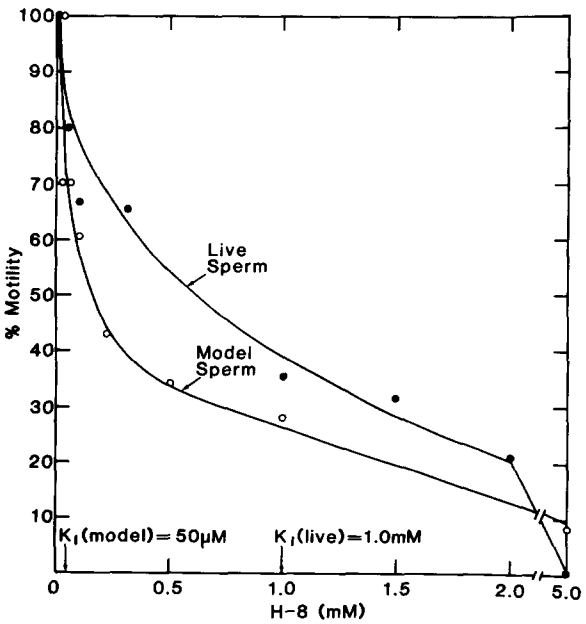

Figure 1. Effect of H-8 on motility of live vs. reactivated sperm. Live sperm or modeled sperm reactivated with 5×10^{-7} M cAMP and 100 μM ATP were incubated in the presence of H-8 at the concentrations detailed in the figure. Percent motility was assessed for 3 min starting 2 min after drug addition. K_i values were calculated by log-logit analysis from the motility data.

High Resolution Two-dimensional PAGE

Phosphorylation reactions were terminated with an equal volume of 2-[*N*-cyclohexylamino]ethane-sulfonic acid (CHES) SDS isoelectric focusing sample buffer (50 mM CHES, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, pH 9.5), then boiled for 10 min in a 100°C bath and DNA was removed by centrifugation at 200,000 *g* for 2 h at 12°C. Comparison of urea with SDS solubilization demonstrated severe (>80%) losses of axokinin when the samples were solubilized with 8 M urea. Two-dimensional PAGE was performed as previously described (17) using the multiple gel casting system of Anderson and Anderson (1, 2) as modified by Health Products, Inc. (MegaDalt; South Haven, MI). The first dimension was isoelectric focusing using mixed pH 3.5–10 ampholines (2:1 ratio of LKB/Pharmacia ampholines, respectively). The second dimension was SDS PAGE on 10–20% slab gels. All gels were stained with the silver-based color stain (14), then equilibrated with acetic acid/glycerol/methanol/water (10:3:30:57) before drying. Removal of the carbonate color development solution (14) was found to be essential to prevent cracking of the gel during drying. ^{32}P incorporation was detected by autoradiography using Kodak XS-5 film and DuPont Quanta III enhancer screens (DuPont Co., Wilmington, DE). Autoradiographic signals were quantitated by digital image processing (12) using known ^{32}P standards.

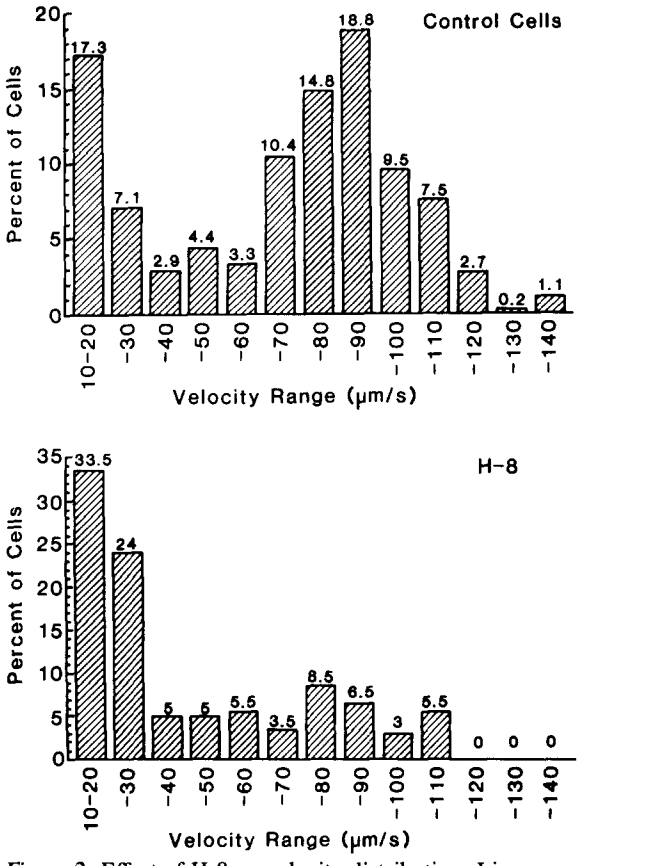

Figure 2. Effect of H-8 on velocity distribution. Live sperm were treated with or without 1.5 mM H-8 for 3 min, then motility was analyzed by digital image processing as described in Materials and Methods. Histograms were then constructed of velocity (in 10 μm/s intervals) versus the percent of the total motile population present in that range. Control and H-8–treated groups represent 452 and 152 sperm, respectively.

Results

Effect of H-8 on Motility

H-8 inhibits motility of live dog sperm in a dose-dependent manner (Fig. 1). Complete inhibition of motility was observed with 5 mM drug. Half-maximal inhibition was achieved with 1 mM H-8 (calculated by linear regression of the log of H-8 concentration vs. logit of the percent motility).

Digital image analysis of motility of the live sperm inhibited with subsaturating (1.5 mM) levels of H-8 demonstrated that other parameters of motility diminished significantly with drug treatment. Examination of the velocity distribution (Fig. 2) demonstrated that 65% were swimming at 60 μm/s or faster in the absence of drug, whereas only 27% were swimming in the same velocity range in its presence. A similar effect was noted for the linearity of the cell trajectories (Fig. 3). Control cells swam with much straighter trajectories (72.1% with linearity ≥6) than drug-treated cells (only 14.9% within the same linearity range). Thus the cells that continue to swim with forward progression in the presence of drug display slower velocity as well as less straighter trajectories than non-drug-treated control cells.

In reactivated models, H-8 inhibited motility at lower drug concentrations than in live cells. The half-maximal inhibition of motility in reactivated sperm (50 μm) was 20 times lower than in live sperm. Complete inhibition of motility in the reactivated cells was not observed under these conditions but >90% inhibition was observed at 5 mM. Velocity parameters were not measurable in the reactivated cells due to sticking of the cells to the coverslip and microscope slide (see Materials and Methods).

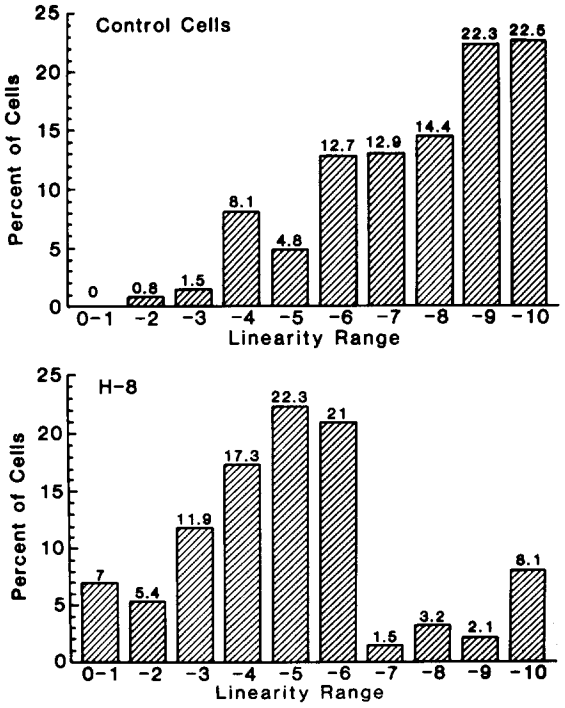

Figure 3. Effect of H-8 on linearity distribution. Live sperm were treated with H-8 and motility analyzed as described in Fig. 2. Histograms were then constructed of linearity versus the percent of the total motile population present in that range. Linearity is calculated by dividing the straight line distance by the curvolinear distance (× 10) for each cell. The larger the value for linearity, the straighter the trajectory of the sperm. Cell numbers are the same as Fig. 2.

H-8 Inhibition of Protein Phosphorylation

Since H-8 inhibits cyclic nucleotide–dependent protein kinases (7) the inhibition of motility by this drug should be coupled to inhibition of protein phosphorylation. This was examined by reactivating sperm with [^{32}P]ATP and determining the effect of 500 μM H-8 on cAMP-stimulated phosphate incorporation. Such reactivations were analyzed by high resolution polyacrylamide gel electrophoresis and autoradiography. As can be seen in Fig. 4, cAMP produced a marked stimulation in phosphorylation of axokinin (indicated by *ax* in each panel) as well as other peptides in the sperm. Inclusion of H-8 resulted in a marked inhibition in the phosphorylation of not only axokinin but nearly all other peptides to levels below that observed with cAMP. Of the major phosphopeptides, only a string of basic proteins at 40 kD was not inhibited to a great degree by H-8. A quantitative presentation of these effects is represented in Table I.

H-8 inhibits both cAMP- and cGMP-dependent protein kinases (7). To determine whether the inhibition of motility and protein phosphorylation by H-8 was in fact due to the inhibition of cAMP-dependent protein kinase, the activity of pure catalytic subunit of cAMP-dependent protein kinase in response to H-8 was determined under reactivation conditions in Hepes reactivation buffer using 100 μM [^{32}P]ATP and histone (Sigma type 2B, Sigma Chemical Co., St. Louis, MO) as substrates. Log-logit analysis of the data (Fig. 5) demonstrated that H-8 inhibited pure catalytic subunit with a K_i of 32 μM. This compares very favorably with the K_i of 50 μM observed for the inhibition of motility by H-8 in the reactivated model.

Previous studies (17) demonstrated that cAMP-dependent protein phosphorylation in the NP-40 extract was required for reactivation of sperm motility. Whether H-8 inhibits protein phosphorylation in the isolated NP-40 extract was examined by incubating isolated extract for 2 min with [^{32}P]ATP and cAMP with and without 500 μM H-8. As a reference, Fig. 6 (upper panel) depicts the entire silver stained two-dimensional protein pattern of the isolated NP-40 extract. The lower panel shows the corresponding autoradiogram of the same sample after phosphorylation for 2 min with [^{32}P]ATP and cAMP. The only major peptide phosphorylated under these reactivation conditions is axokinin (56 kD, pI ~6) as depicted by the arrow in both panels. Under these conditions axokinin represents 97% of the incorporated phosphate, as determined by digital image processing. Fig. 7 demonstrates that axokinin phosphorylation in the isolated NP-40 extract is reduced over 500-fold in the absence of cAMP (middle panel). Similarly, 500 μM H-8 reduced axokinin phosphorylation in the presence of cAMP by 85% (lower panel). This degree of reduction by H-8 would be predicted on a theoretical basis since 10 times the calculated K_i should result in an 87% inhibition of phosphorylation.

H-8 Does Not Inhibit Motility of Sperm Reactivated with Phosphorylated Axokinin

Comparison of the cAMP-dependent phosphoprotein pattern in reactivated sperm (Fig. 4) with that observed in the iso-

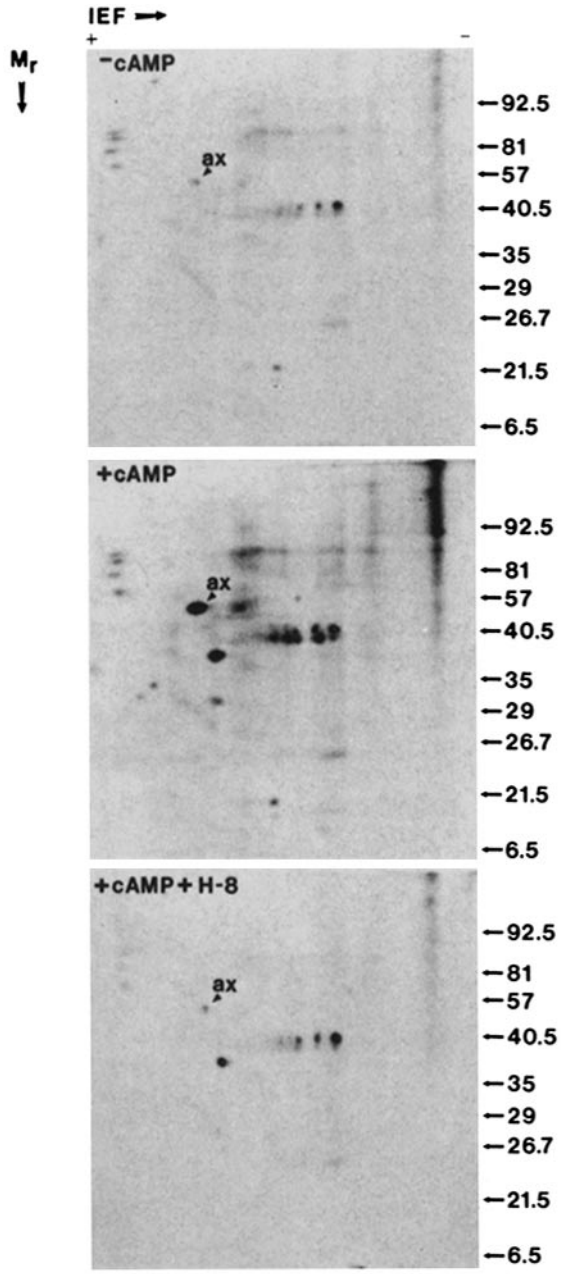

Figure 4. Inhibition of protein phosphorylation in reactivated dog sperm by H-8. Permeabilized dog sperm were reactivated for 2 min with 100 μM [^{32}P]ATP in the absence (−cAMP) or presence (+cAMP) of 5 × 10^{-7} M cAMP or cAMP and 500 μM H-8 (+cAMP + H-8). Reactions were terminated and analyzed by high resolution two-dimensional polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. Each panel represents an identical number of sperm and autoradiographs that were exposed, photographed, and developed identically. Relative isoelectric migration (*IEF*) is represented horizontally from acidic on the left to basic on the right. Molecular mass in kilodaltons is represented vertically with the position of molecular weight standards indicated on the right of each panel. *ax*, position of axokinin.

lated NP-40 extract (Fig. 6) demonstrated the large number of phosphoproteins present in sperm (but not in the NP-40 extract) that were stimulated by this nucleotide. Although the phosphorylation of axokinin is known to be required for reactivation of motility (17), we sought to determine to what

Table I. Quantitation of Selected cAMP-dependent Phosphopeptides Blocked by H-8

Peptide	Phosphorylation	Counts/spot	Ratio (as % of + cAMP)
Axokinin	+ cAMP	1,406,000	–
	− cAMP	54,200	3.8
	+ cAMP + H-8	40,000	2.8
38 kD	+ cAMP	766,300	–
	− cAMP	4,900	0.6
	+ cAMP + H-8	125,100	16.3
40 kD (Most basic peptide)	+ cAMP	290,800	–
	− cAMP	188,900	65.0
	+ cAMP + H-8	200,600	69.0

The autoradiograms depicted in Fig. 4 were quantitated by digital image processing as described in Materials and Methods. Axokinin is indicated in Fig. 4 by *ax*. The 38-kD peptide is the major cAMP-dependent peptide slightly to the right and below axokinin. The 40-kD most basic peptide is the right-most peptide of string of major signals just below and to the far left of axokinin.

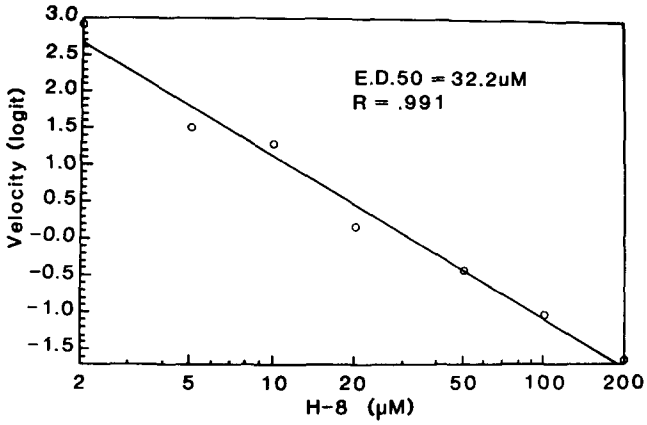

Figure 5. Inhibition of pure catalytic subunit of cAMP-dependent protein kinase by H-8 under reactivation conditions. Pure catalytic subunit of cAMP-dependent protein kinase was assayed in the presence of increasing quantities of H-8 under reactivation conditions using histone (Sigma type II) as substrate and 100 μM [^{32}P]ATP. Data are presented as drug concentration versus percent inhibition on log-logit ordinates. The value for K_i was calculated by linear regression analysis of the log of H-8 concentration versus logit of percent catalytic subunit activity remaining at each drug concentration. Each point was measured in triplicate.

extent these other phosphoproteins may be involved in the stimulation and/or maintenance of motility. To answer this question sperm were preincubated in the presence or absence of 5 mM H-8 and then models of each group were prepared. Each group was then reactivated in the presence or absence of cAMP and/or H-8 as indicated in the table. As summarized in Table II, upon reactivation, the H-8–treated cells showed no movement, whereas cells not immobilized with H-8 (control) showed good reactivation. After centrifugation to remove the NP-40 extract, the control group displayed significantly lower but still measurable reactivation. The H-8–treated group continued to remain completely immotile. Reactivation of both groups was achieved by reconstitution with nonphosphorylated extract plus cAMP if H-8 was omitted during reactivation. In the presence of H-8, reactivation with nonphosphorylated extract and cAMP was reduced

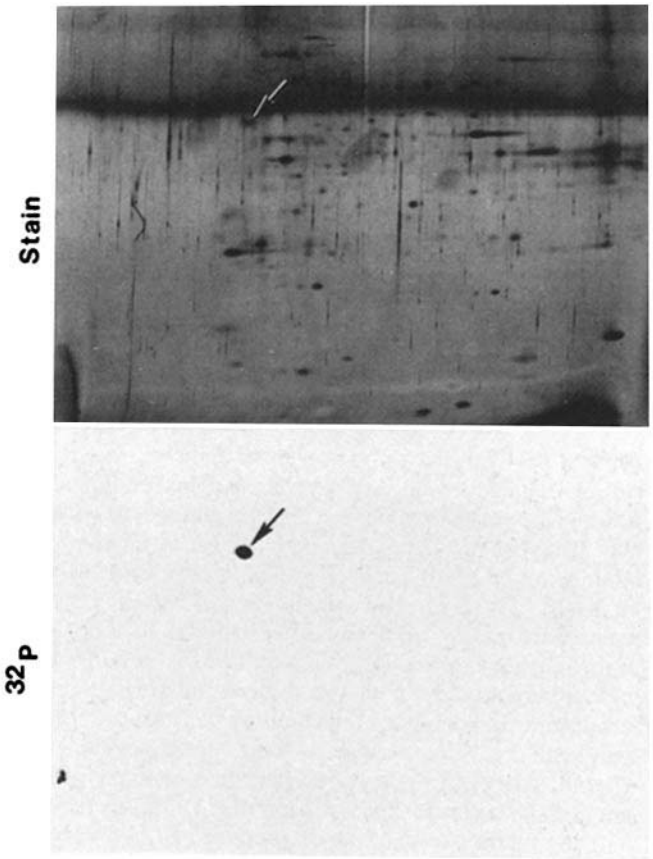

Figure 6. Protein and phosphoprotein pattern of isolated NP-40 extract of dog sperm. An NP-40 extract of sperm was phosphorylated for 2 min with [^{32}P]ATP and cAMP then analyzed by high resolution two-dimensional polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. *Upper panel*, silver-stained protein pattern of the extract. *Lower panel*, corresponding autoradiograph of the same sample. The position of axokinin is marked by the arrow in both panels.

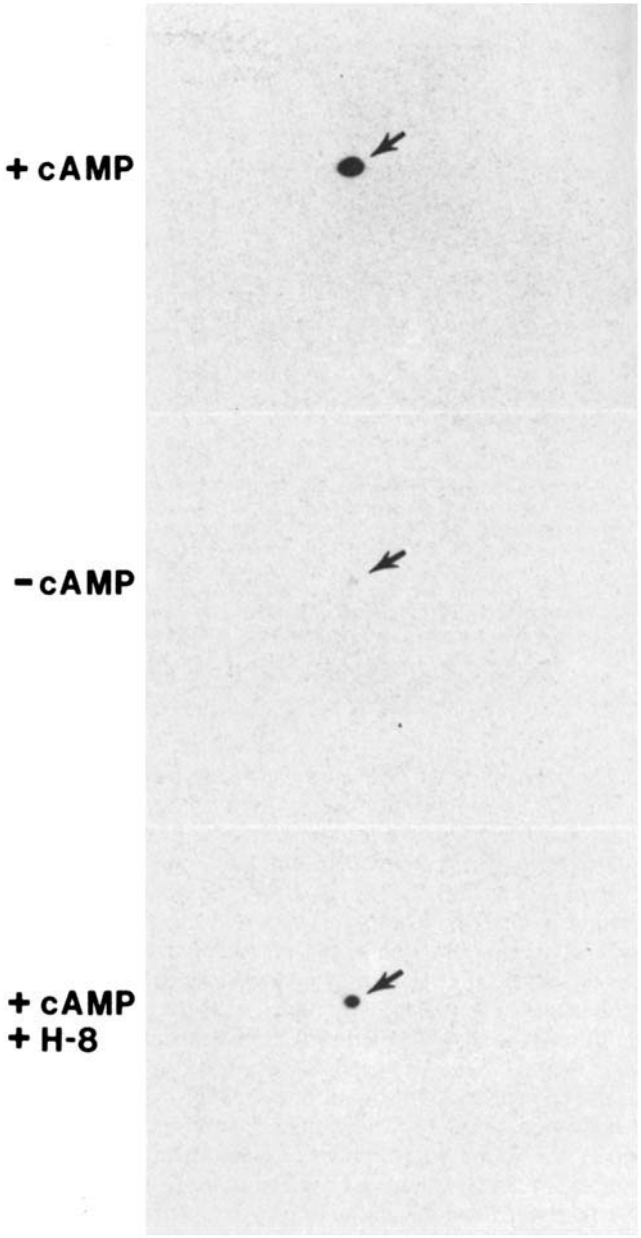

Figure 7. Inhibition of protein phosphorylation in the NP-40 extract by H-8. An isolated NP-40 extract was phosphorylated for 2 min with 100 μM [^{32}P]ATP in the absence (−cAMP) or presence (+cAMP) of 5×10^{-7} M cAMP or cAMP and 500 μM H-8 (+cAMP + H-8). Reactions were terminated and analyzed by high resolution two-dimensional polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. Each panel represents an identical quantity of extract protein. The position of axokinin is marked by the arrow in each panel.

fivefold in the control group and blocked almost completely in the H-8–treated group. It should be noted that the low reactivation achieved in the cells pretreated with H-8 was significantly lower ($P = 0.057$) than that observed in the corresponding control group. On the other hand, when prephosphorylated extract was used both the control and H-8–treated groups showed good reactivation even in the presence of H-8. Thus in the absence of cAMP-dependent protein phosphorylation (as inhibited by H-8), the presence of phosphorylated axokinin is sufficient to fully reactivate sperm to the starting level of flagellar activity originally present in the unfractionated models. It should be noted that in both pretreatment groups, the degree of reactivation achieved with phosphorylated extract in the presence of H-8 was only slightly lower ($P = 0.084$) than that achieved in its absence, suggesting little if any effect of this level of H-8 on motility-coupled dynein ATPase activity.

Discussion

The results presented here suggest that phosphorylation of proteins in the NP-40 extract of sperm are sufficient for flagellar reactivation to occur in permeabilized models of dog sperm. This conclusion is predicated on the observation that full reactivation of sperm occurred even in the presence of saturating amounts of H-8 if prephosphorylated extract was used. Thus in the absence of any contribution of cyclic nucleotide–dependent phosphorylation by the cells the only requirement for flagellar reactivation is that the NP-40 extract is in a phosphorylated state. Since axokinin represents 97% of the cAMP-dependent phosphoprotein present in the NP-40 extract, we suggest that phosphorylated axokinin is necessary and sufficient for reactivation of flagellar motility.

Table II. Axokinin Phosphorylation Is Sufficient for Flagellar Reactivation

Cells	Reactivation cAMP	Reactivation H-8	% Motility Control	% Motility Pre-H-8
Model before extraction	+	*	57.4 ± 5.8	0.0 ± 0.0
Extracted sperm	+	−	3.1 ± 1.5	0.0 ± 0.0
Reconstituted sperm with				
Nonphosphorylated extract	+	−	40.4 ± 3.0	37.2 ± 5.8
	+	+	8.3 ± 3.5	1.1 ± 1.1
Phosphorylated extract	−	−	74.3 ± 4.3	67.2 ± 6.1
	−	+	69.5 ± 3.5	53.8 ± 8.1

Washed dog sperm were incubated for 1.25 h at room temperature in the absence or presence of 5 mM H-8. Models were prepared of each and then extracted as described in Materials and Methods and reactivated in the presence or absence of H-8 using extract that had been either prephosphorylated or not, as described below. An NP-40 extract was prepared from normal washed dog sperm and split into two aliquots. One aliquot was phosphorylated for 2 min with ATP and cAMP, then heat-treated, centrifuged, and dialyzed to obtain the prephosphorylated extract. The other aliquot was heat treated, centrifuged, and dialyzed as above (without the phosphorylation incubation) to obtain the nonphosphorylated extract. Both aliquots were used to reactivate the extracted dog sperm described above in the absence or presence of H-8 as indicated. Percent motility was quantitated by video image analysis as detailed in Materials and Methods.

* Control cells were reactivated in the absence of H-8; pretreated cells were reactivated in the presence of H-8.

The presence of the cAMP second messenger cascade system in sperm and the stimulatory role for this nucleotide on sperm have been known for some time (6, 8, 10). However, substrates for the protein kinases that may be involved in the control of flagellar motility have only recently been discovered. Brandt and Hoskins (3) reported the presence of a ~55-kD phosphoprotein in bovine epididymal sperm. The degree of phosphorylation by cAMP-dependent protein kinase in vitro was inversely proportional to the level of sperm motility at the time of homogenization. Tash and Means (15) were the first to identify candidate phosphoproteins involved in the control of sperm motility by use of [^{32}P]ATP in the reactivation solution. Under these conditions several proteins were found whose phosphorylation state was closely coupled to cAMP-stimulated flagellar motility. Ishiguro et al. (9) discovered that the ability of cAMP to stimulate motility of sea urchin and star fish sperm models depended on the presence of material solubilized by detergent treatment. A similar requirement was noted for dog sperm (17). Examination of the detergent extract of dog sperm led to the identification of only five phosphoproteins. One of these, a 56-kD peptide, represented the most likely candidate as it was the only cAMP-dependent phosphoprotein common to extracts of human, dog, and sea urchin sperm as well as ciliary sources such as retina and trachea. The flagellar-stimulating activity of this protein, named axokinin, was also heat stable in all tissues in which it was found. Axokinin was absent in nonflagellar sources such as brain, heart, skeletal muscle, and liver.

While the evidence suggested cAMP-dependent phosphorylation of axokinin was required for motility to occur, the extent to which the other sperm cAMP-dependent phosphoproteins might be involved in the control of flagellar motility remained unanswered. An examination of the temporal relationship between basal and cAMP-stimulated motility revealed that the maximal effect of cAMP on the initiation of motility occurred within the first 2 min of reactivation (17). Indeed, if phosphorylation is carried out under these conditions of reactivation (as opposed to the 5 min originally reported by Tash et al. [17]), then axokinin represents 97% of the cAMP-stimulated phosphate incorporated in the isolated NP-40 extract (Fig. 6). Other cAMP-stimulated phosphoproteins become detectable but only after incubation times longer than required for cAMP to exert its stimulatory effect on motility (17). Therefore, it seemed reasonable to assume (until purified axokinin can be tested) that axokinin is the primary phosphoprotein in the NP-40 extract responsible for conferring cAMP-dependence upon flagellar motility.

The availability of the isoquinolinesulfonamide, H-8, afforded the opportunity to test whether non-extractable sperm proteins that are phosphorylated by cAMP- (or cGMP-) dependent protein kinase might also be required for cAMP-stimulated flagellar motility. The fact that the compound can traverse the plasma membrane made it possible to confirm, now using live cells, the observations made with the protein inhibitor of cAMP-dependent protein kinase in reactivated models (9, 17). Since H-8 inhibits both cAMP- and cGMP-dependent kinases (7), the values for half-maximal inhibition of the purified enzyme (Fig. 5) were compared to those for inhibition of motility in the reactivated models (Fig. 1). The fact that the drug inhibited both motility and the pure enzyme with similar K_i values suggests that the mode of inhibition of motility by the drug is via its effect on protein phosphorylation. Such a conclusion was supported by the marked decline in protein phosphorylation produced by H-8 in whole models (Fig. 4) as well as isolated NP-40 extract (Fig. 6). Furthermore, the degree to which H-8 inhibited protein phosphorylation was near the predicted value based upon the calculated K_i of H-8 for catalytic subunit and the concentration of drug added to the phosphorylation mixtures (10 times the K_i).

When prephosphorylated extract is used, no other cAMP-dependent phosphoproteins need be present to obtain full reactivation of flagellar motility. This conclusion is based on two assumptions: (*a*) the presence of 2 mM H-8 is sufficient to prevent essentially all endogenous phosphorylation catalyzed by cAMP- (or cGMP-) dependent protein kinase, and (*b*) H-8 does not inhibit other enzymes required for motility. Concerning the first assumption, in vitro assay of cAMP-dependent protein kinase demonstrated a K_i of H-8 for the enzyme of 32 μM under reactivation conditions. The level of H-8 in the reactivation experiments using prephosphorylated extract represented sufficient inhibitor to block 96% of the endogenous catalytic subunit activity based on standard competitive inhibitor kinetics (5). We conclude, therefore, that essentially all of the endogenous catalytic subunit activity was inhibited. With reference to the second assumption, that H-8 does not have a significant effect on other enzymes involved in the control of flagellar motility; the motility achieved in the presence of H-8 with prephosphorylated extract was not significantly different from the motility achieved without inhibitor or with the requisite control using cAMP and nonphosphorylated extract.

In conclusion, our results suggest that axokinin is a pivotal component of the regulatory machinery of flagellar motility. For flagellar motility to occur, axokinin must be present in a phosphorylated state. Furthermore, this phosphorylation is catalyzed by cAMP-dependent protein kinase. Finally, axo-

kinin appears to be the only protein that need be phosphorylated in a cAMP-dependent manner for flagellar motility to be expressed.

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