BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 231–237 (1998)
ARTICLE NO. RC977937

Identification of Flagellar Proteins That Initiate the Activation of Sperm Motility *in Vivo*

Gerácimo E. Bracho, Jennifer J. Fritch, and Joseph S. Tash
Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7401

Received December 3, 1997

Protein phosphorylation appears to be a necessary step in the intracellular signaling pathway that initiates the activation of sperm motility. Activation of live immotile sea urchin sperm produced rapid, time-dependent increased phosphorylation on proteins of 32, 45, 130, and 500 kDa. Fractionation of immotile and motile sperm indicated that these motility-related phosphoproteins are associated with flagella. These proteins showed greater phosphorylation in the flagellar fraction from motile sperm, suggesting that subcellular boundaries are in place to keep protein kinases and their substrates spatially separated. Solubility properties suggest that these proteins are the heavy chain and smaller subunits of sea urchin sperm dynein which are phosphorylated *in vivo* to initiate activation of motility. This also suggests that phosphorylation of only these few proteins, out of the nearly 100 phosphorylations known to occur in the basic axoneme, appears to be associated with the early signaling pathways of motility activation in intact sperm.

Previous studies have shown that protein phosphorylation is associated with the initiation and modulation of sperm motility. The importance of cAMP in the initiation and maintenance of sperm motility has long been known [(1, 2), reviewed in Tash (3) and Tash & Bracho (4)]. Detergent-permeabilized sperm reactivated with exogenous ^{32}P-ATP demonstrated that increased levels of cellular cAMP showed concomitant activation of protein kinases as well as increased phosphorylation of endogenous proteins (5, 6). Putative soluble factors involved in motility activation have been identified in a variety of species including mammalian sperm (3, 5, 7-12). However, the contribution of soluble factors for motility initiation remains unknown, as models examined thus far begin with sperm that have already undergone variable degrees of activation (5, 10, 13-15).

In characterizing the mechanisms of sperm activation by soluble factors, we discovered that detergent alone had a major stimulatory effect on reactivation of permeabilized dog sperm (3). This suggested that detergent might allow interaction of signal transduction pathway enzymes and substrates that might not normally occur in vivo. However, to add exogenous ^{32}P-ATP for sperm activation and subsequent analysis of phosphorylation, membrane compartmentalization had to be destroyed. The problem is further magnified by individual sperm diversity and sperm collection procedures and storage conditions which allow variable levels of motility expression prior to experimental activation (16-18). This makes the identification of primary regulatory components difficult. It is also possible that some regulatory proteins could undergo rapid phosphorylation and that their dephosphorylation might be extremely slow or non-existent. In this case, subsequent attempts to label these proteins with ^{32}P in reactivation experiments would either fail or give highly variable levels of incorporation, depending on the extent of 'background' motility and phosphorylation prior to preparation of the model.

We sought to eliminate these problems by using the sperm collection method previously described (19), which minimize sperm exposure to normal sea water during spawning and sperm activation during storage prior to experimental activation. Furthermore, the experiment was performed with live rather than permeabilized sperm, to eliminate potential fortuitous kinase-substrate interactions created by lost compartmentalization. In this paper, we present results indicating that immotile sperm, as expected, contain low levels of flagellar protein phosphorylation. More importantly, results also show that only a few flagellar proteins are phosphorylated in the early biochemical events of sperm activation.

MATERIALS AND METHODS

Materials. Sea urchins (*Stronglyocentrotus purpuratus* and *Lytechinus pictus*) were from Marinus (Long Beach, CA). Polyclonal

0006-291X/98 $25.00

anti-phosphoserine (anti pS) and anti-phosphothreonine (anti pT) and monoclonal anti-phosphotyrosine (anti pY) antibodies were from Zymed Corporation (San Francisco, CA). Peroxidase conjugated secondary antibody was from Pierce (Rockford, IL). Chemiluminescence reagents for Western analysis were from Amersham Corp. (Arlington Heights, IL). N-stearate-DLIEEAASRIVDAVIEQVKAAGAY (S-Ht31) and N-stearate DLIEEAASRPVDAVIEQVKAAGAY (S-Ht31-P) were generous gifts from Dr. Daniel W. Carr, Veterans Affairs Medical Center and Oregon Health Sciences University, Portland, OR. SDS-PAGE reagents and protein markers were from Bio-Rad (Richmond, CA). All other reagents were analytical grade.

Buffers. The composition of artificial sea water (ASW), sperm activating buffer (HSW), sperm storage buffer (SSB), and MES sperm storage buffer (MSSB) was previously described (19). In addition, the following buffers were used. Dynein ATPase activity assay buffer (AB), consisted of 20 mM imidazole-HCl pH 7.2, 10% sucrose, 100 mM NaCl, 0.5 mM EGTA, 0.1% 2-mercaptoethanol, 6 mM $MgCl_2$, and 0.03% Brij 35. Cyclic AMP-dependent protein kinase (PKA) activity buffer contained 10 mM calcium chloride, 100 μM ATP and 50 mM Tris-HCl pH 7.0 (20). Sperm protein serine/threonine phosphatase (PPS) activity buffer was previously described (21). Other buffers are described where appropriate.

Sperm preparation. Sperm were prepared as previously described (19). A pool of sperm from several animals was made to minimize individual variations. Sperm in MSSB were always adjusted to a final concentration of 1×10^8 cells/ml prior to experimentation and stored at 4-5°C.

Sperm motility activation and protein phosphorylation determination. Immotile sperm in MSSB were activated by dilution into activation buffer (HSW) as previously described (19). Analysis of motility was performed as previously described (19). Protein phosphorylation was performed with immotile sperm in MSSB activated by dilution into HSW. Activation was terminated by adding 4X SDS-PAGE sample buffer (22) at 0, 30 and 60 sec. Zero time points were established by premixing sample buffer and HSW prior to addition of sperm, thus ensuring that a true zero was achieved. Controls were performed with immotile sperm in MSSB diluted into MSSB and addition of 4X SDS-PAGE sample buffer as previously described. Samples were spun at 200000g for 2 h at 4°C to remove DNA. Supernatants were used for protein electrophoresis and Western analysis.

Sperm and flagella fractionation. Sperm were fractionated at 4°C essentially as previously described (23), with slight modification. Two identical sperm samples in MSSB were harvested by 15 min centrifugation at 4000g and the buffer discarded. One sample was activated by suspending the sperm pellet in HSW and incubating at room temperature for 15 min. The other sample was kept immotile by suspending in MSSB and incubating under the same conditions. Both sperm samples were again harvested as described above. The two samples were then suspended in a sucrose solution (20 % w/v sucrose, 20 mM imidazole-HCl pH 6.0, 0.1% 2-mercaptoethanol, 0.5 mM EGTA, 2.5 mM $MgCl_2$, 5 mM benzamidine, 1 mM PMSF, and 1 μg/ml each of the following: leupeptin, pepstatin A, and aprotinin A) and fractionated into heads and flagella by 12 strokes of a Potter-Elvehjem tissue grinder with PTFE pestle. Heads were pelleted by centrifugation as before, and flagella in the supernatant were carefully decanted to avoid head contamination, and saved. The head pellets were suspended in sucrose, homogenized and centrifuged again. Flagella fractions were again carefully decanted and combined with the first fractions. Heads were suspended in sucrose solution, homogenized with 12 strokes and stored at −80°C for analysis. The isolated flagella fractions were diluted with equal volumes of extraction buffer (20 mM imidazole-HCl pH 7.2, 200 mM NaCl, 0.1% 2-mercaptoethanol, 0.5 mM EGTA, 7 mM $MgCl_2$, 1% w/v CHAPS and protease inhibitors as described above), homogenized with 12 strokes and centrifuged at 245000g for 1 h. The supernatants, which contained most of the PKA and PPS activity, were saved and stored frozen at −80°C until analysis. The flagellar pellets were suspended in extraction buffer containing 600 mM NaCl but no detergent, homogenized with 12 strokes and centrifuged again at 245000g for 1h. Under these extraction conditions, these supernatants contain the bulk of ATPase activity normally associated with outer arm dynein (23). The supernatants were saved and stored frozen at −80°C for analysis. The pellets, which contained primarily axonemal proteins, were suspended in sucrose solution, homogenized with 12 strokes and stored at −80°C for analysis.

Electrophoresis and Western analysis. Western analysis (24) was performed on nitrocellulose membranes containing proteins separated by 5-15% gradient SDS polyacrylamide electrophoresis (22). All incubations were at room temperature on a rocker platform. Membranes were blocked for 1h with 2% (w/v) BSA in TTBS buffer [20 mM Tris-HCl pH 7.6, 137 mM NaCl, and 0.1% (w/v) Tween 20]. Membranes were then washed and incubated with primary antibody (1:10,000 in TTBS) for 1h. Washes were repeated and membranes incubated with peroxidase conjugated-secondary antibody (1:5,000 in TTBS) for 1 h. Immunocomplexes were detected in the washed membranes after 1 min reactions with ECL reagent and X-ray film exposure for 10-90 sec. Western controls were incubated for 15 min with 2 mM of free phosphorylated amino acid prior to primary antibody incubation.

Protein, ATPase, PKA, and PPase activity determinations. Protein concentrations were measured in triplicate by the microprotein assay of Bradford (25) with bovine serum albumin as the standard. Dynein ATPase activity was assayed in triplicate by quantitation of the release of inorganic phosphate at room temperature (22°C) in a 96-well ELISA plate by a malachite green assay as described by Harder et al. (26). PKA activity was determined in triplicate at 30°C by measuring incorporation of ^{32}P from ^{32}P-ATP to a peptide representing residues 81 to 99 of the phosphorylation site of type II regulatory subunit of rat skeletal muscle PKA (27) as previously described (20). PPS activity was determined in triplicate at 30°C by measuring the dephosphorylation of the ^{32}P-phosphorylated RII peptide described above (21).

RESULTS AND DISCUSSION

A new method of immotile sea urchin sperm preparation (19) was used to investigate early protein phosphorylation events associated with the initiation of sperm motility. This study addressed the variable levels of motility that detergent permeabilized sperm allow before experimental activation occurs (5, 10, 13-15). Experiments were performed with live immotile sperm, to eliminate the potential problems created by fortuitous kinase-substrate interactions caused by destroyed compartmentalization. Because ^{32}P-ATP could not be used to examine internal protein phosphorylations of live sperm, these changes were examined using anti phosphoamino acid antibodies (28, 29).

We found that immotile sperm, as expected, had extremely low levels of protein phosphorylation. However, the most remarkable finding was that activation produced very rapid, time-dependent increased phosphorylation of only a few sperm proteins, as shown in Figure 1. This suggests that phosphorylation of only these few proteins, from the nearly one hundred phosphorylations known to occur in the basic axoneme (30), appears to be associated with the early biochemical events of motility activation in intact sperm.

Figure 1 shows Western analysis with anti pS anti-

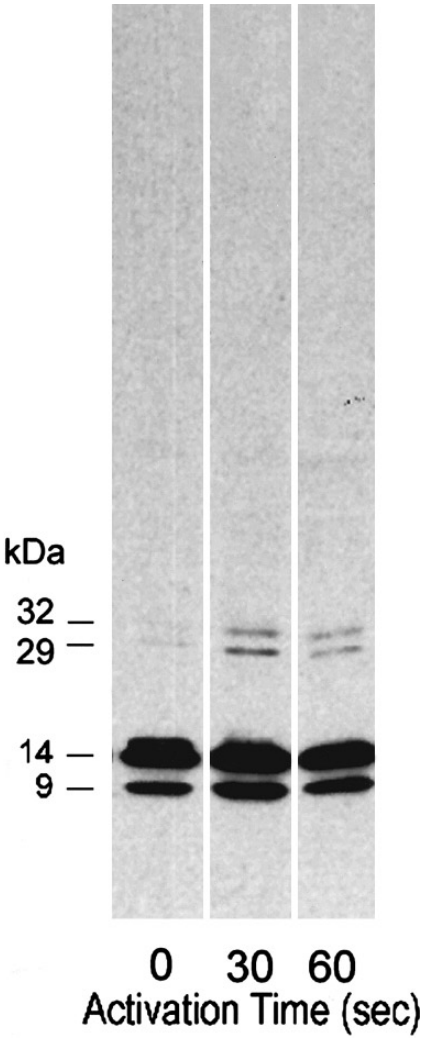

FIG. 1. Serine phosphorylation of *S. purpuratus* sperm proteins during initiation of motility. Immotile sperm in MSSB were activated by dilution into HSW. Activation was terminated by addition of 4X SDS-PAGE sample buffer at 0, 30, and 60 sec. Zero controls were obtained by dilution of MSSB sperm into HSW premixed with SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and examined for phosphorylation by Western analysis with anti pS antibodies. Comparable results were obtained using *L. pictus* sperm. See Materials and Methods for other details.

body and indicates one set of proteins migrating at 29-32 kDa predominantly phosphorylated at serine residues. Analysis with anti pT antibody identified a set of higher molecular weight proteins phosphorylated at threonine residues, as described below. Anti pY antibody reactivity was limited to the same set of proteins identified with anti pS antibody, but substantially weaker (data not shown). Overall, these results were in sharp contrast to those obtained with reactivated sperm, which were characterized by the presence of numerous phosphoproteins that appear to be unrelated to motility (5, 14, 31, 32). Immunostaining was blocked by the presence of the homologous phosphorylated free amino acid but not by the others, which is suggestive of the specificity of the reactions.

To determine whether the proteins that are phosphorylated during the initiation of activation are directly related to motility, fractions were prepared from immotile and motile sperm and examined for the presence of proteins phosphorylated on serine and threonine residues by Western analysis. Examination of phosphorylated tyrosine was not performed due to the weak immunoreactivity observed with whole sperm. To compare the relative degree of phosphorylation of fractions from immotile and motile sperm, gels were run using the same amount of protein per lane. Figs. 2 and 3, show analysis with anti pS and pT antibody, respectively. These figures indicate that only fractions from whole sperm and heads showed the same low M_r proteins (9-14 kDa) noted in Fig. 1. This indicates that these phosphoproteins were associated with the heads, and did not appear to be correlated with motility because their degrees of phosphorylation did not change with time of sperm activation.

Examination of the anti pS blot (Fig. 2), indicates that the 29-32 kDa motility-associated phosphoproteins noted in Fig. 1, were not detected at this protein loading in the sperm or heads fractions. However, they were clearly visible in the flagellar, HS extract and axonemal fractions (indicated by arrows). In flagella prepared from motile sperm (right lane), the 29 and 32 kDa doublet showed greater phosphorylation than flagella prepared from immotile sperm (left lane). Similarly, phosphorylation of a 45 kDa protein also increased with activation but to a lesser extent. Under these conditions no significant serine phosphorylation was observed in the PPS/PKA fractions. In examining the HS fractions, we speculate that the 32 kDa protein might be associated with dynein because it was partially extracted from flagella with a 0.6 M NaCl buffer normally used to partially extract outer arm dynein (23). In these fractions, phosphorylation of the 32 kDa protein was greater than that observed in the flagellar fractions. However, the difference in phosphorylation between fractions from immotile and motile sperm appeared to be lost. This might indicate a loss of compartmentalization due to the detergent demembranation and high salt buffer extraction of flagella. Loss of compartmentalization in the flagellar fractions would allow interaction of kinases and substrates regardless of whether samples originated from immotile or motile sperm. In addition, the creatine kinase shuttle system has been shown to be partly bound to flagella, and it requires non-ionic detergent for extraction (33-35). Thus, a portion of the ATP regenerating system could also be present to promote additional phosphorylation during flagellar extraction. In the high salt-extracted axonemes, protein bands were also noted at 29, 32 and 45 kDa, but again no apparent difference in phosphory-

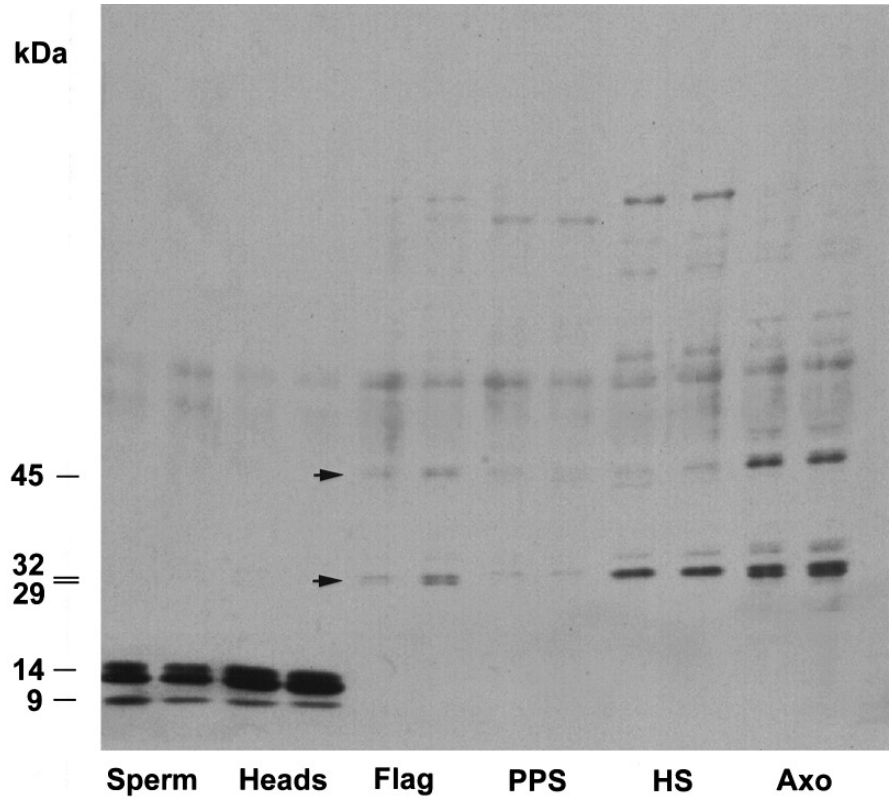

FIG. 2. Protein serine phosphorylation in fractions from immotile and motile *S. purpuratus* sperm. Immotile sperm in MSSB were activated by dilution into HSW or kept immotile by dilution into MSSB. The immotile and motile sperm were fractionated as described in Materials and Methods. Fractions were immediately mixed with 4X SDS-PAGE sample buffer. Electrophoresis was performed by loading 2 μg of protein per lane. Samples are presented in pairs. The left lane indicates the fraction from immotile sperm and the right lane indicates the corresponding fraction from motile sperm. Arrows in the flagellar fractions indicate the pS-containing proteins that showed an increase in phosphorylation in motile sperm. In the flagellar and axonemal fractions, the lower band migrating as a doublet at 29 and 32 kDa appeared to be the same proteins identified in the whole sperm fraction (Fig. 1). Comparable results were obtained using *L. pictus* sperm. See Materials and Methods for other details.

lation levels between immotile and motile sperm was observed, for reasons indicated previously.

Western analysis of immotile and motile sperm fractions with anti pT antibody is presented in Figure 3. In contrast to the 29-32 kDa doublet identified with anti pS antibody, only the 32 kDa protein was detected with anti pT antibody in the flagellar, HS extract and axonemal fractions. The 45 kDa protein detected with anti pS antibody (Fig. 2) was again observed with anti pT antibody, but reactivity was relatively lower. In addition, two new proteins were identified at 41 kDa and at 130 kDa in the PPS/PKA and flagellar fractions, respectively, that appeared to be associated with the initiation of motility. The 130 kDa protein was not extracted with 0.6 M NaCl, implying that it is likely to be tightly bound to the axoneme. As observed earlier for the pS-containing motility proteins (Fig. 2), the changes in phosphorylation of the 130 kDa protein observed in flagella were also lost during fractionation. This 130 kDa phosphoprotein may be related to the 138 kDa phosphoprotein regulatory subunit of inner arm dynein identified in *Chlamydomonas* (36). The solubility properties of the 130 kDa protein identified in sea urchin flagella are consistent with it being an inner arm dynein subunit. The enrichment of proteins in the flagellar, HS and axonemal fractions revealed additional phosphoproteins that were not observed in whole sperm fractions. A 160 kDa protein containing predominantly pT was observed in the flagellar and HS extracts, but its degree of phosphorylation did not change with activation. Another pT 68 kDa protein in flagella that appeared to diminish in phosphorylation during activation was also found in the HS extract. Finally, an increase in pT was observed in proteins that appeared large enough ($\sim$500 kDa) to represent dynein heavy chains in the flagellar, HS extract, and axonemal fractions (HC, Fig. 3). Similar phosphorylation profiles were found in both *S. purpuratus* and *L. pictus* sperm, suggesting that motility regulatory proteins are the same in both species.

These data suggest that soluble sperm proteins are not a major target for phosphorylation during initiation

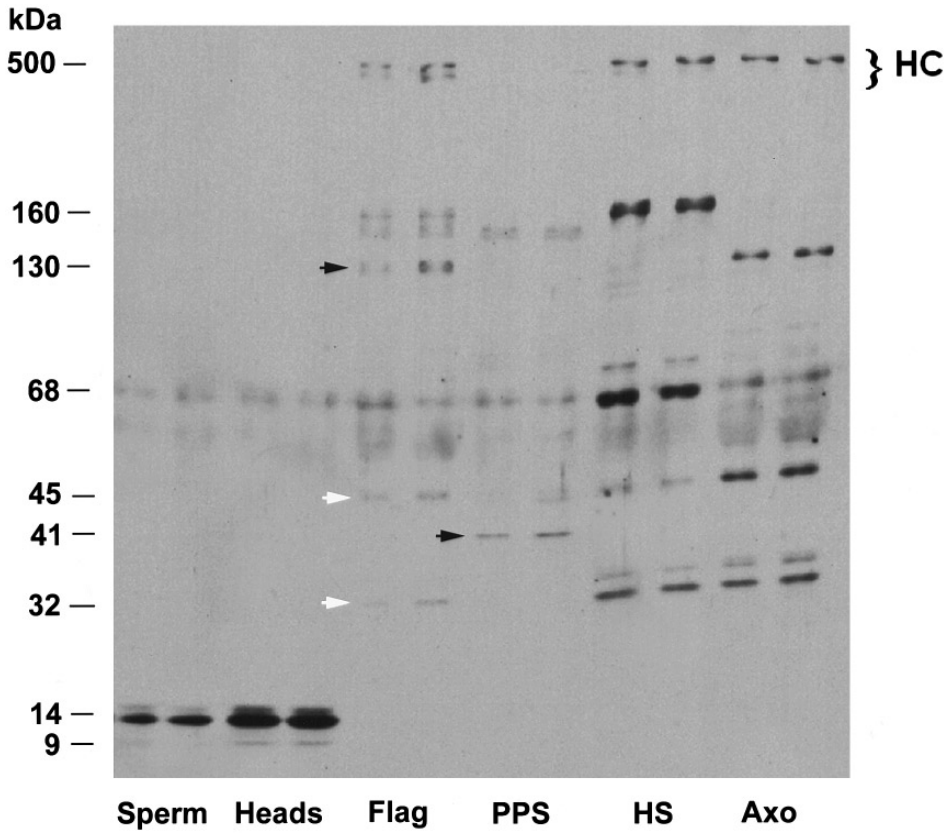

FIG. 3. Protein threonine phosphorylation in fractions from immotile and motile *S. purpuratus* sperm. The experiments were run as indicated in Fig. 2, but examination of phosphorylation was performed with anti pT antibodies. White arrows in the flagellar fractions indicate the same motility related proteins that were identified with anti pS antibody. The black arrows at 130 kDa in the flagellar fraction and 41 kDa in the PPS fraction indicate pT-containing proteins that showed an increase in phosphorylation with motility activation. An increase in pT was also observed in proteins that appeared to represent dynein heavy chains (HC) in the flagellar, HS extract, and axonemal fractions. Comparable results were obtained using *L. pictus* sperm. See Materials and Methods for other details.

of motility. Previous studies have identified the type II regulatory subunit (RII) of PKA as a major soluble phosphoprotein in mammalian sperm (5, 7, 8). Although RII is present in flagella of sea urchin sperm (37, 38), we did not find serine phosphorylation of proteins corresponding to RII (55-56 kDa) in any of the sperm fractions during initiation of motility. Therefore, it appears that RII phosphorylation is not relevant to the early events of motility activation in live sea urchin sperm.

Collectively, this extraction and analysis support the idea that in live immotile sea urchin sperm, protein kinases and their substrates are spatially separated and brought together only when the biochemical events of motility activation are initiated. This idea is further considered in combination with several conclusions that we have drawn from the results presented in this paper. First, the degree of protein phosphorylation in live immotile sea urchin sperm, and perhaps other sperm, is extremely low. Second, phosphorylation of only a limited set of flagellar proteins appears to be necessary to trigger the initial biochemical events of motility activation. Third, the few regulatory proteins identified here all have extractability properties consistent with being associated with sperm dyneins. Our interpretation of how all of these events occur is emerging as a complex process that is regulated in many ways, for the following reasons. In live immotile sperm, kinases and substrates appear to be spatially separated and brought together only when initiation of motility commences. How this occurs is currently a matter of speculation. However, one way to accomplish this is by the same mechanism that allows the subcellular distribution of kinases and phosphatases to be determined by association with targeting subunits [reviewed in (39)] (40-42). Formation of kinase-phosphatase signaling complexes has been shown to modulate the phosphorylation state of specific target substrates (43, 44). Regulation and specificity of PKA, in particular, appears to be determined by anchoring to specific subcellular sites through the interaction of the regulatory subunit with *A K*inase *A*nchoring *P*roteins or AKAPs (reviewed in (45)). Recent work indicates that interaction of the regulatory subunit of PKA with sperm

AKAPs appears to be a key regulator of motility. Vijayaraghavan et al. (46) have used membrane-permeable AKAP peptides to arrest motility in vigorously motile human, monkey and bovine sperm. In addition, they found that inhibition of PKA catalytic activity had little effect on basal motility or motility stimulated by agents previously thought to work via PKA activation. On the contrary, we found that their most inhibitory peptide (S-Ht31) stimulated motility activation of live immotile sea urchin sperm (data not shown). The reasons for the differential effects of the AKAP peptide on these sperm are unknown. However, these differences could in part be attributed to structural differences in these sperm or to diversity of AKAPs. In regard to structure, sea urchin sperm unlike mammalian sperm contain no fibrous sheath, and localization of mitochondria is limited to the base of the flagella. Therefore, sea urchin sperm could have a distribution of AKAPs that have a unique contribution depending on target specificity. Disruption of the AKAP complex could result in the release of unequal ratios of phosphatase and kinase activity in the different sperm. Diversity is supported by the identification of a developmentally regulated human sperm AKAP (S-AKAP84) localized to the mitochondria (47), the cloning and characterization of a rat testis AKAP (AKAP220) that associates with peroxisomes (48), and the cloning and characterization of a testis-specific, developmentally regulated AKAP (TAKAP80) present on the fibrous sheath of rat sperm (49).

Finally, we propose that a role for phosphatases in these events is the dephosphorylation of kinase-phosphorylated substrates to keep a steady state of phosphorylation and maintain motility. Examination of activation time points in Fig. 1, indicates that the degree of phosphorylation at 60 sec appears to be lower than at 30 sec. A simplified interpretation of this is that the earliest biochemical event in motility activation is very rapid kinase phosphorylation (30 sec or less) of a few regulatory flagellar proteins. The presence of phosphorylated proteins would then stimulate the activity of phosphatases, as indicated by the considerable amount of dephosphorylation observed at 60 sec.

ACKNOWLEDGMENTS

This study was supported by Grant NAG-2-1016 from NASA (J.S.T.) and Grants HD-33994 from NIH to the Center for Reproductive Sciences and HD-02528 to the Mental Retardation & Human Development Research Center. We thank Dr. Daniel Carr for the generous gift of S-Ht31 and S-Ht31-P peptides. We also thank Dr. Bill Kinsey for critical reading of the manuscript.

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