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Regulation of Sperm Flagellar Motility by Calcium and cAMP-Dependent Phosphorylation

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There is substantial evidence that cAMP-dependent phosphorylation is involved in the activation of motility of spermatozoa as they are released from storage in the male reproductive tract. This evidence includes observations that in vivo activation of motility can be inhibited by protein kinase inhibitors, can be reversed by protein phosphatase treatment of demembranated spermatozoa, and is associated with phosphorylation of sperm proteins, and observations that spermatozoa that have not been activated in vivo can be activated in vitro by cAMP-dependent phosphorylation. Activation in vivo can often be triggered by conditions that increase intracellular pH, but the relevance of this to in vivo activation under natural conditions and the steps between pH increase and cAMP increase have not been fully established. The relationships between changes in the protein substrates for cAMP-dependent phosphorylation and changes in axonemal function are still unknown.

Sperm chemotaxis to egg secretions is widespread; in the sea urchin *Arbacia*, the egg jelly peptide resact has been identified as a chemoattractant. Response to chemoattractants involves changes in assymmetry of flagellar bending waves, and similar changes in asymmetry can be produced in vitro by increases in $[Ca^{++}]$. Temporal changes in resact receptor occupancy might lead to transient changes in intracellular $[Ca^{++}]$ and the asymmetry of flagellar bending, but many links in this hypothetical sequence remain to be established.

Both of these signalling systems offer immediate opportunities for investigations of biochemical pathways leading to easily assayable biological responses. However, complications resulting from interactions between these two systems need to be considered.

Key words: spermatozoa, sperm flagella, intracellular pH, chemotaxis, guanylate cyclase, cGMP, sea urchin spermatozoa, adenylate cyclase, cAMP-dependent protein kinase, calmodulin

Two aspects of regulation of flagellar motility in spermatozoa will be discussed in this article, based on a review of recent literature supplemented with mention of some unpublished work, primarily from my own laboratory. These studies can be

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viewed in either of two ways: as studies that take advantage of observations of motility to report on regulatory events, or as studies that utilize known regulatory biochemistry to dissect the still-largely-unknown mechanisms involved in the motility of the flagellar axoneme.

ACTIVATION

A general problem in the life of spermatozoa is the need to rapidly activate the functions of the cell following a period of energy-conserving storage. Two such functions are the activation of motility and the preparation of the cell for fusion with an egg surface, involving capacitation or an acrosome reaction. I will only discuss the problem of activation of motility, and I will focus primarily on systems in which new results have been obtained since the reviews by Garbers and Kopf [1] and Tash and Means [2], and in particular on a system that I have been working with, the spermatozoa of the lower chordate, *Ciona*. Recent reviews of sea urchin sperm activation include consideration of the other aspects of activation [3,4].

Sperm Flagellar Motility can be Activated In Vitro by cAMP-Dependent Phosphorylation

Ciona spermatozoa can be obtained in concentrated suspension from the sperm duct by dissecting the animal. For detailed examination of the motility of live spermatozoa, the spermatozoa need to be diluted by a factor of 1,000 or more. However, this dilution can activate motility, making it difficult to ask questions about the state of activation of the spermatozoa. It is more convenient to assay the state of activation with an in vitro assay, which involves demembranation with Triton X-100 followed by transfer to a suitable medium containing $MgATP^{--}$ for reactivation of motility.

Spermatozoa taken directly from the sperm duct and assayed in this manner show *no* motility. These spermatozoa can be activated in vitro by incubation with cyclic AMP [5,6]. The results depend on the duration of incubation with cyclic AMP [7]. The effect of cyclic AMP is certainly the result of activation of an endogenous cAMP-dependent protein kinase:

It requires ATP;

It can be replicated by exogenous protein kinase catalytic subunit in the absence of cAMP [5];

It can be inhibited by protein kinase inhibitors and reversed by treatment of the spermatozoa with a protein phosphatase preparation [5,7];

It is accompanied by cAMP-stimulated incorporation of phosphate into sperm proteins [5].

Activation of motility and protein phosphorylation are affected similarly by KCl concentration and cAMP concentration [5].

A similar requirement for in vitro activation with cAMP has been shown for trout spermatozoa [8]. With sea urchin spermatozoa, the situation is more complicated. Demembranated spermatozoa from a widely studied species, *Strongylocentrotus purpuratus*, are usually fully motile without any explicit activation process, in vivo or in vitro, and demonstration of dependence of motility on cAMP-dependent phosphorylation requires special demembranation conditions [16]. With *Lytechinus pictus* spermatozoa, nearly all samples of concentrated spermatozoa showed only

partial activity after demembranation unless activated in vitro by incubation with cAMP [6]. Using Japanese sea urchin species, Ishigura et al [9] prepared a fraction from the detergent supernatant from demembranated spermatozoa that conferred cAMP sensitivity on the demembranated spermatozoa. A dependence on cAMP was also obtained with aged spermatozoa [10]. Ishiguro et al presented evidence that a protein factor from the supernatant was required in addition to cAMP-dependent protein kinase, and evidence that the phosphorylated form of this factor could partially activate motility when mixed with demembranated spermatozoa in the presence of protein kinase inhibitor [9].

The earliest observations suggesting a role for cAMP-dependent phosphorylation in the activation of sperm motility were made with mammalian spermatozoa [11,12]. Reactivated dog spermatozoa, obtained by demembranation of washed, ejaculated spermatozoa, normally show relatively low-percentage motility, which can be greatly increased by addition of cAMP to the reactivation solutions [13]. A heat-stable 56-kD protein found in the detergent supernatant, given the name axokinin, has been identified as the significant substrate for cAMP-dependent phosphorylation in this system [14]. After incubation of the detergent supernatant with cAMP, this axokinin-containing supernatant was able to fully activate the motility of demembranated spermatozoa even in the presence of concentrations of the kinase inhibitor, H8, adequate to prevent cAMP-induced activation [15].

Tash et al [14] presented evidence for the presence of a protein with axokinin-like properties in detergent extracts of sea urchin and human spermatozoa and *Chlamydomonas* flagella, as well as dog spermatozoa, and their results with axokinin are consistent with the results of Ishiguro et al already mentioned [9]. However, with *Ciona* spermatozoa, although a necessary supernatant factor in addition to the cAMP-dependent protein kinase has been indicated [5], this factor appears to be heat sensitive (inactivated by 3 min at 100°C) and could not be replaced with a detergent extract from sea urchin spermatozoa. All attempts to activate *Ciona* spermatozoa in the presence of protein kinase inhibitors by addition of previously phosphorylated supernatant have failed [5].

In some cases, a partial activation of motility in vitro can be obtained without cAMP incubation by incubation with trypsin. This works well in trout [17] and sea urchin [6] but not in *Ciona* spermatozoa [18]. Presumably trypsin can in some cases destroy a regulatory mechanism that is restraining movement in the absence of cAMP-dependent phosphorylation.

In Vivo Activation of *Ciona* Spermatozoa Also Involves Phosphorylation by a cAMP-Dependent Protein Kinase

Ciona spermatozoa can be activated in vivo by treatments that probably cause an increase in internal pH. For example, spermatozoa can be diluted 1:25 with a solution of 90% 0.5 M NaCl, 10% sea water, and 80 mM histidine at pH 8.4. After 20 sec, this suspension can be diluted 1:10 with demembranation solution and the reactivated motility can be assayed. This procedure normally gives 100% motility. The seawater in the activation solution is not essential, but it appears to improve the quality of the reactivated motility. A convenient control solution is 0.5 M NaCl, 10 mM $NaHCO_3$, and 5 mM HEPES buffer, at pH 7.5. Dilution with this solution before demembranation does not activate motility. Evidence that this in vivo activation also

involves cAMP-dependent phosphorylation is provided by the following observations:

Both in vitro and in vivo activation can be similarly reversed by treatment with protein phosphatase [7];

In vivo activation decreases subsequent in vitro cAMP-dependent phosphorylation of sperm proteins [6];

In vivo activation can be inhibited by the protein kinase inhibitor, H8, introduced by Hidaka et al [19].

The experiments with H8 showed that addition of 0.4 mM H8 was sufficient to completely inhibit in vitro activation under standard conditions. For in vivo activation, concentrated spermatozoa were diluted 1:25 with control solution containing 1 mM H8. After 20 sec, this mixture was mixed with an equal volume of in vivo activation solution containing 1 mM H8; 20 sec later, this mixture was diluted with demembranation solution. After dilution with reactivation solution, essentially no reactivated motility was obtained; the same procedure, with the H8 omitted, gave 100% reactivated motility. H8 appears to penetrate these spermatozoa very rapidly; no time course for its effect could be measured. Addition of 1 mM H8 *after* in vivo activation did not reverse activation [16].

In vivo activation of trout spermatozoa has been shown to be associated with a transient increase in cAMP content and increased adenylate kinase activity [20,21] and can similarly be inhibited by 1 mM H8 [22]. Evidence that a 15-kD axonemal polypeptide is the major axonemal substrate for in vitro cAMP-dependent phosphorylation and is also phosphorylated during in vivo activation has been presented [23].

Reversal of the state of activation of sperm flagellar motility by the protein kinase inhibitor H8 has been described in dog spermatozoa [15], suggesting that protein phosphatases are still active in these sperm preparations. Reversal of activation of sea urchin spermatozoa by protein phosphatases has been described by Takahashi et al [24]. Decreases in in vitro phosphorylation correlated with bull sperm activation were found by Brandt and Hoskins [12]. Increases in sea urchin sperm cAMP content and adenylate cylase activity associated with conditions that activate motility have also been described [25].

An Increase in Internal pH Is a Common Trigger for Sperm Motility Activation

The question of the mechanism by which sperm motility and metabolism are repressed during storage and activated upon release has probably received more attention in sea urchins than in any other system. The most recent work solidly establishes internal pH as a critical controlling variable [26,27]. Changes in internal pH have been demonstrated by measurements of the partitioning of radioactive or fluorescent amines between the medium and the sperm cytoplasm, and by ^{31}P-NMR. A pH increase associated with activating conditions is the result of two or more factors, including the removal of a volatile inhibitor (probably CO_2; [28]) and alkalinization by a Na^+:H^+ exchange requiring the presence of external sodium ions ([29,30]; but see also [31]). Lee [32] demonstrated that this Na^+:H^+ exchange is a property of the membranes of isolated flagella and is inhibited when the membrane is depolarized by increasing external K^+ ion concentration. The reactivated motility of demembranated sea urchin spermatozoa is strongly inhibited by reducing the pH from normal values (above 8.0) down to 7.5 [33]. It has been suggested that this may be a

sufficient explanation for pH-dependent regulation of in vivo motility [26], especially in *S purpuratus*, where demembranated spermatozoa are usually fully motile without any explicit activation process. Nevertheless, a large increase in internal cAMP concentration can be demonstrated when these spermatozoa are exposed to conditions that cause an increase in internal pH [25], and recent work has suggested that cAMP-dependent phosphorylation is also involved in activation of motility in these spermatozoa [16]. Activating effects of internal pH increase, independent of changes in cAMP concentration, have also been reported for bull spermatozoa [36].

The procedure for assaying in vivo activation of *Ciona* spermatozoa by using demembranation and reactivation to report the state of activation is very easy to use to ask additional questions about, for example, the role of inorganic ions in activation. Such experiments reveal that K^+ is a potent inhibitor of activation, and that this inhibition can in some cases be reversed by Ca^{++} [16]. In trout spermatozoa, in vivo activation is inhibited by K^+ ions, and this phenomenon is believed to be utilized by maintaining a high K^+ ion concentration in semen during storage in the male [20,34]. Inhibitory effects of K^+ ion can be counteracted by Ca^{++} ions [35]. It is not yet clear whether all of these effects may be interpretable in terms of changes in internal pH.

Unanswered Questions

There are many unanswered questions about this signalling system. With the possible exceptions of CO_2 removal from sea urchin spermatozoa and K^+ removal from trout spermatozoa, we don't really know what the environmental signal for activation is and whether the high pH conditions that have been used to obtain in vivo activation are similar to or are just bypassing the normal signal. In another interesting system, spermatozoa of the horseshoe crab, *Limulus*, are activated only by a peptide obtained from eggs, and this response does not appear to be mediated by an increase in internal pH [37,38]. In this system, motility can also be activated by removing Zn^{++} from the spermatozoa with chelating agents [39]; this has also been reported for starfish spermatozoa [40].

We don't know how may successive signalling events occur within the cell leading to activation of adenylate cyclase and an increase in cAMP concentration that activates the cAMP-dependent protein kinase. Evidence for the presence of the α-subunits of GTP-binding regulatory proteins (G proteins) in sea urchin and other spermatozoa has been reported [41,42], suggesting that the same signal transduction systems that regulate adenylate cyclase in other systems may be operating here. It has not been established whether these G proteins are involved in activation of motility or other sperm functions.

We don't know what functional changes in the flagellar axoneme occur as a result of cAMP-dependent phosphorylation, although some progress has been made in characterizing motility parameters that depend on cAMP-dependent phosphorylation [7] and in identifying the substrates for this phosphorylation that can be resolved as discrete polypeptide bands by polyacrylamide gel electrophoresis and autoradiography [5,14,15,23]. One interesting candidate substrate appears to be the 15-kD band described by Morisawa and Hayashi [23], but nothing else is known about this protein. In *Ciona*, preliminary work suggested that a dynein high molecular weight polypeptide was phosphorylated during both in vitro and in vivo activation, but with a relatively low specific activity [5].

Spermatozoa such as those of *Ciona* and trout appear to be very promising for future study of these problems, because there is a quick and easy assay for the state of activation, and because it is easy to produce both the completely inactive and the completely active states. Trout spermatozoa can easily be obtained in the large volumes needed for biochemical studies, and several groups are now working with these spermatozoa.

CHEMOTAXIS AND THE REGULATION OF WAVEFORM ASYMMETRY BY Ca^{++}

Sperm chemotaxis to substances released by eggs or accessory cells has been known in plants for over a century and has been demonstrated for many species of marine invertebrates during the past two decades [43]. The most detailed observations on sperm behavior during chemotaxis have been made with spermatozoa of the coelenterate, *Tubularia*. In spite of extensive efforts, identification of the attractant molecules involved in animal sperm chemotaxis has been difficult, but one molecule has now been identified.

Resact Is the Chemoattractant for *Arbacia* Spermatozoa

Resact was originally identified as a peptide from egg jelly of the sea urchin, *Arbacia punctulata*, that activated the respiration of homologous spermatozoa at low pHs. The sequence of this 14-amino acid peptide has been established [44]. It has now been found to be a potent chemoattractant for these spermatozoa [45], with sensitivity detectable with concentrations as low as 1 nM.

Resact Causes a Transient cGMP Increase

The resact receptor in the sperm membrane also appears to be the sperm membrane guanylate cyclase. Resact binding stimulates the activity of this enzyme, followed by a dephosphorylation of the enzyme that down-regulates its activity [46–49]. The result is a transient increase in cGMP concentration inside the spermatozoa [50].

Chemotaxis Is Suppressed by Reducing Ca^{++} Concentration

Chemotaxis of bracken fern spermatozoids was shown to be inhibited by EDTA or other Ca^{++} chelators [51]. Suppression of sperm chemotaxis in the hydroid, *Tubularia* [52], and in the siphonophore, *Muggiaea* [53], was obtained with Ca^{++}-free artificial seawater. With *Arbacia* spermatozoa, artificial seawater solutions with reduced Ca^{++} concentration showed progressively less response to resact, with little or no response below 0.5 mM Ca^{++} [45].

Phospholipid Methylation May be Involved in Chemotactic Signalling

The chemotactic response of starfish spermatozoa to (unidentified) chemoattractants from ovaries of homologous starfish species is enhanced by the transmethylase inhibitor homocysteine. Exposure of spermatozoa to the chemoattractant peptides caused a large decrease in phospholipid methylation, leading to suggestions that phospholipid methylation may be important in the response to temporal changes in receptor occupancy [54].

Chemotactic Response Involves Increases in the Asymmetry of Flagellar Bending Waves

Tubularia spermatozoa normally swim in paths with low curvature. In the vicinity of a source of chemoattractant (egg extracts) they make sharp turns, caused by transient increases in the asymmetry of flagellar bending waves [55]. These sharp turns are almost always in the same direction as the steady-state curvature of the sperm swimming path, suggesting that the spermatozoa always respond by increasing their basal level of asymmetry in only one direction relative to the internal structures of the sperm flagellum.

Increases in Asymmetry of Flagellar Bending Waves are Produced In Vitro by Increases in Ca^{++} Ion Concentration

Effects of Ca^{++} ion concentration on the asymmetry of sperm flagellar bending waves have been studied in detail with ATP-reactivated, demembranated, spermatozoa from *S purpuratus* [56,57]. These studies show that the effect of Ca^{++} ion is highly specific; the changes in asymmetry are accompanied by little or no change in other parameters of bending waves, such as frequency, mean bend angle, or wavelength. Similar effects of Ca^{++} ion on bending wave asymmetry have also been observed with species showing chemotactic response: *Arbacia* [45], *Ciona*, and *Tubularia* [16].

Calmodulin may be Involved in Mediating the Response to Ca^{++}

Extraction of calmodulin from *Lytechinus pictus* spermatozoa by exposure to millimolar [Ca^{++}] and calmodulin-binding compounds such as trifluoroperazine or Triton X-100 decreases the asymmetry of subsequent flagellar bending. This asymmetry can be restored by addition of bovine brain calmodulin, even at very low (ca 10^{-9} M) Ca^{++} concentrations [58]. However, the response of sperm bending wave asymmetry to Ca^{++} concentration is a gradual response extending over about four decades of Ca^{++} concentration [56,57], and calmodulin may be mediating only the response in the upper portion of this range [16].

Unanswered Questions

Theoretical considerations suggest that chemotactic detection of directional information most likely results from detection of temporal changes in receptor occupancy as a spermatozoon moves through a concentration gradient, rather than simultaneous comparison of receptor occupancy at different locations on the sperm surface [59,60]. This has not yet been established experimentally for sperm chemotaxis.

A most simple-minded model for sperm chemotaxis would be that a temporal decrease in receptor occupancy causes a transient increase in internal Ca^{++} concentration, lasting only a fraction of a second, causing a turn that increases the probability that the spermatozoon will resume swimming in a direction that will carry it toward higher attractant concentrations. Whether sperm chemotaxis is too precise to be explained by such a simple mechanism has not been determined, except in the case of bracken spermatozoids [51,61], where increased precision may be explained by the presence of multiple flagella. Unfortunately, this model is not supported by the direct measurements of Schackmann and Chock [62], which show that an *increase* in intracellular Ca^{++} concentration in sea urchin spermatozoa is induced by the analogous peptide, speract.

Although the in vitro responses of reactivated, demembranated spermatozoa to increases in Ca^{++} concentration are well documented, it has not been established that in vivo changes in internal Ca^{++} concentration are associated with asymmetric flagellar bending waves. An attempt to demonstrate this with a monoclonal antibody known to elevate internal Ca^{++} concentration [63] gave negative results [64].

The mechanism of axonemal response to Ca^{++} is unknown. The response curve for asymmetry vs Ca^{++} concentration [16,56,57] does not resemble that expected for any simple Ca^{++}-binding receptor such as calmodulin. The detailed changes in flagellar waveform when the bending waves become asymmetric can be satisfactorily explained by a "biased baseline" mechanism, in which symmetric flagellar bending waves are propagated along an axoneme having a static, Ca^{++}-regulated, curvature [65]. This mechanism is supported by observations that the static curvature of flagella in which dynein-based oscillation has been inhibited with 5 μM vanadate can be altered by changes in Ca^{++} concentration [66]. It supports speculations that axonemes contain novel mechanisms for Ca^{++}-induced changes in configuration that may be independent of the dynein-driven active sliding process.

INTERACTIONS BETWEEN ACTIVATION AND ASYMMETRY REGULATION

Lytechinus spermatozoa, demembranated without calmodulin extraction or cAMP incubation, are predominantly quiescent in reactivation solutions containing 1 mM $MgATP^{--}$. They can be activated in vitro to produce asymmetric bending waves *either* by incubation with cAMP *or* by calmodulin extraction [6,7,58]. *Both* treatments are required to obtain symmetric bending waves. Quiescence appears to be just an extreme manifestation of conditions that produce asymmetric bending waves. This interpretation is also consistent with the observation that quiescence can be induced by high Ca^{++} concentrations [67] if spermatozoa have not been calmodulin-extracted, even after extensive incubation with cAMP, and it is consistent with a probable role for calcium in intermittent quiescence of live spermatozoa [68].

Live spermatozoa that are nonmotile at low pH ($\sim$6.5) or in the presence of an egg jelly fucose sulfate glycoconjugate can be activated by the egg jelly peptides speract and resact [44], and increases in internal cAMP concentration are observed under these conditions [25]. This effect is mediated by activation of K^+ channels which leads to membrane repolarization and reactivation of the voltage-sensitive Na^+:H^+ exchanger [69]. There is therefore a possible correlation between the suggestion that during chemotaxis resact removal causes internal Ca^{++} concentration to increase, causing increased asymmetry of flagellar bending waves, and an activating effect of resact addition that might result from reduction in internal Ca^{++} concentration and reduced bend asymmetry. However, this is an extremely tenuous connection which should be considered to be no more than a suggestion for possible future investigations.

The two preceding paragraphs do not reflect an integrated understanding of the interactions between the systems regulating activation and waveform asymmetry in sea urchin spermatozoa. Rather they indicate a need for caution in trying to interpret these as two independent regulatory systems.

A more direct interaction between effects of calcium and cAMP-dependent phosphorylation has been described in two ciliary systems. In *Paramecium*, where direct evidence for regulation of a bending pattern by intracellular Ca^{++} was first

obtained, the reverse beating induced in detergent-extracted cell models by Ca^{++} concentrations above 0.5 μM could be reversed by addition of cAMP [70]. Evidence that this was an effect of cAMP-dependent phosphorylation was provided, but in contrast to effects on sperm activation, cGMP was as effective as cAMP. Similarly, in the mussel, *Mytilus*, lateral cilia on the gill filaments can be arrested by Ca^{++}, and motility can be restored by cAMP-dependent phosphorylation [71,72]. In both of these cases, there is no obvious analogy to the activation of sperm motility after release from storage, and this may permit cAMP-dependent phosphorylation to be utilized in a different manner. Nevertheless, both the ciliary systems and the sperm systems clearly show that effects in opposite directions are produced by increases in Ca^{++} concentration and by cAMP-dependent phosphorylation. Examination of the substrates for cAMP-dependent phosphorylation of cilia has begun in several laboratories [71–74].

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