

Hyperactivation of mammalian spermatozoa: function and regulation

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Hyperactivation is a movement pattern observed in spermatozoa at the site and time of fertilization in mammals. It may be critical to the success of fertilization, because it enhances the ability of spermatozoa to detach from the wall of the oviduct, to move around in the labyrinthine lumen of the oviduct, to penetrate mucous substances and, finally, to penetrate the zona pellucida of the oocyte. The movement of hyperactivated spermatozoa appears different under different physical conditions and in different species, but basically it involves an increase in flagellar bend amplitude and beat asymmetry. Presumably, there is a signal or signals in the oviduct to initiate hyperactivation at the appropriate time; however, none has yet been identified. There is evidence that the source of the signal is follicular fluid, yet spermatozoa are known to hyperactivate before ovulation would release the fluid into the oviduct. Although the signal transduction cascade regulating hyperactivation remains to be described completely, it is clear that calcium ions interact with the axoneme of the flagellum to switch on hyperactivation. The process may also involve increases in intracellular cAMP, which at least is required to support motility in general. Although hyperactivation usually occurs during capacitation, the two events are regulated by different pathways.

Hyperactivated movement of spermatozoa was first reported by Yanagimachi (1969), who observed that, as spermatozoa gained the ability to fertilize oocytes *in vitro*, their flagella began beating more vigorously than before. He proposed that the vigorous beating plays a vital role in the penetration of the zona pellucida.

Yanagimachi's proposal that hyperactivated motility enables spermatozoa to penetrate the zona pellucida was later confirmed by experiments. Stauss *et al.* (1995) used various methods that prevented hyperactivation but did not inhibit the acrosome reaction, and demonstrated that hyperactivated hamster spermatozoa were more successful at penetrating the zonae pellucidae of oocytes *in vitro* than spermatozoa that were not hyperactivated.

Additional functions have been ascribed to hyperactivation. These were based on consideration of the obstacles encountered by spermatozoa in the oviduct. Mucus is present in the isthmus of the oviduct of rabbits (Jansen, 1978), humans (Jansen, 1980), pigs (Suarez *et al.*, 1992) and cattle (Suarez *et al.*, 1997). In many species, spermatozoa also encounter another type of mucus, consisting primarily of hyaluronic acid, while passing through the cumulus oophorus. Mucus increases the

viscosity and elasticity of the aqueous milieu in which spermatozoa swim. Both hamster (Suarez *et al.*, 1991) and mouse (Suarez and Dai, 1992) spermatozoa penetrated viscoelastic substances more effectively when they were hyperactivated; therefore, hyperactivated spermatozoa are likely to be more effective at penetrating oviductal mucus and the cumulus matrix *in vivo*.

The behaviour of hamster and mouse spermatozoa within transilluminated oviducts indicated that hyperactivation enables spermatozoa to move about effectively in the lumen until they encounter the oocyte. Hyperactivated spermatozoa placed on a microscope slide in simple aqueous medium often spend a lot of time swimming in circles, whereas within the oviduct, the walls of which are not hard and flat like those of a slide, hyperactivated spermatozoa appear to cover space effectively in a search pattern. Mouse spermatozoa used the deep flagellar bends characteristic of hyperactivation to turn around within pockets of mucosa and escape out into the central lumen (Suarez and Osman, 1987). Hyperactivated hamster spermatozoa glided rapidly over the mucosal surface of the ampulla (Katz and Yanagimachi, 1980).

In several species of mammal, a reservoir of spermatozoa forms within the isthmus of the oviduct. The reservoir is thought to preserve spermatozoa and ensure that they are in the proper state for fertilization when oocytes enter the oviduct, and is formed when spermatozoa are trapped by

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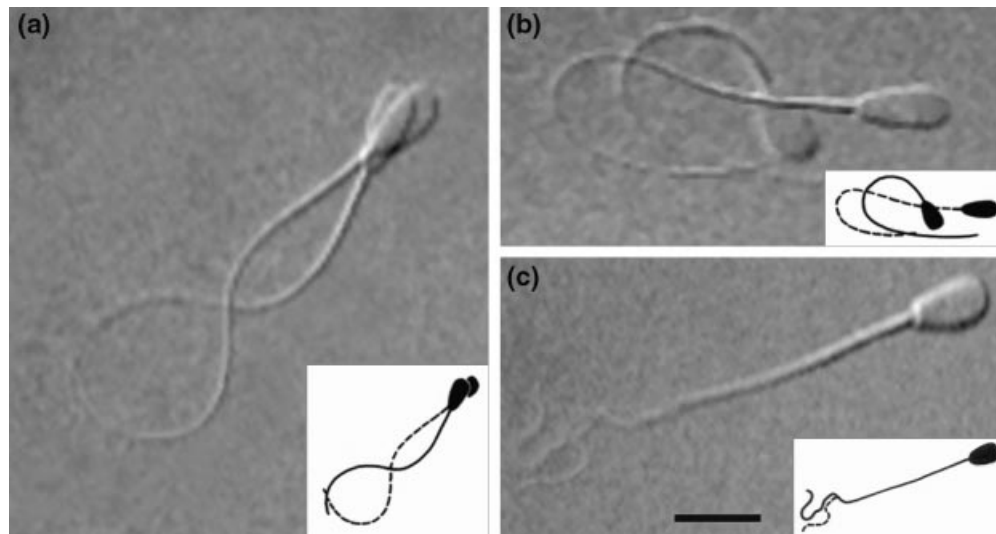


Fig. 1. Video images of swimming patterns of hyperactivated bull spermatozoa on glass slides in shallow chambers approximately 10 μm deep. Spermatozoa were illuminated by flashes from a xenon strobe at 60 Hz. Video frames were captured at 30 Hz by a Dage CCD 72 camera and digitized using a Scion CG-7 imaging board. Each image represents two consecutive video fields spaced 1/60 s apart. Traces of the images are shown in insets. (a) The symmetrical flagellar beat pattern of activated spermatozoa. (b) The asymmetrical flagellar beat pattern of hyperactivated spermatozoa. (c) The progressive swimming of hyperactivated spermatozoa in a thick, viscoelastic solution of long-chain polyacrylamide. Scale bar represents 10 μm .

sticking to the mucosal epithelium. In hamsters, horses and cattle, this interaction is mediated by carbohydrate recognition, that is, a lectin-like molecule on the surface of spermatozoa binds to a specific oligosaccharide moiety on the epithelium (DeMott *et al.*, 1995; Suarez *et al.*, 1998). In cattle, the lectin-like molecule has been identified as PDC-109 (also known as BSP-A1/A2), a protein secreted by the seminal vesicles that coats the heads of ejaculated spermatozoa (Ignatz *et al.*, 2001). Loss or modification of the molecule apparently releases spermatozoa from the epithelium (Ignatz *et al.*, 2001). Release may be assisted by hyperactivation. During examination of the movements of mouse spermatozoa within the oviduct, DeMott and Suarez (1992) noticed that only spermatozoa showing hyperactivated movement pulled away from the epithelium. Hyperactivation may pull spermatozoa off the epithelium as their hold on the oligosaccharide moiety weakens.

Definition of hyperactivation

It is difficult to define hyperactivation precisely and globally by the physical characteristics of the swimming pattern. The pattern of sperm movement varies among species and according to the physical environment in which spermatozoa swim. It is best to define hyperactivated motility in comparison with activated motility for each particular species and to do so for spermatozoa swimming in a physiological liquid medium on a slide. Activated motility is defined by Yanagimachi (1994) as follows. Spermatozoa within the epididymis are

inactive or only weakly motile. When they are released into seminal plasma *in vivo* or into physiological medium *in vitro*, they rapidly begin to swim vigorously in a nearly straight trajectory (Fig. 1a). Hyperactivated motility is defined as the swimming pattern shown by most spermatozoa retrieved from the oviductal ampulla at fertilization. The flagella of hyperactivated spermatozoa are thrown into deeper bends and their beating is usually less symmetrical than that of flagella of activated spermatozoa (Figs 1 and 2). As a result, hyperactivated spermatozoa tend to swim vigorously in circles on a microscope slide. In some species, spermatozoa that are fully hyperactivated complete a circle in two flagellar beats, giving the appearance of swimming in figures-of-eight.

Some authors have described hyperactivation as biphasic, because they observed spermatozoa alternating between the activated and hyperactivated swimming patterns (Johnson *et al.*, 1981; Tessler and Olds-Clarke, 1985). It could be argued that this is the result of hyperactivation being switched on and off. As discussed below, very little is known about the mechanisms that regulate hyperactivation; therefore, it is impossible to know whether hyperactivation is truly biphasic.

One factor affecting the flagellar beating pattern, which results in variation in patterns of activation and hyperactivation among species, is the rate of rotation of spermatozoa about the longitudinal axis (rolling, Fig. 2). Activated rabbit, bull and boar spermatozoa roll rapidly as they swim. Freshly activated hamster spermatozoa roll rarely and appear to glide along on slides in a gently curved path

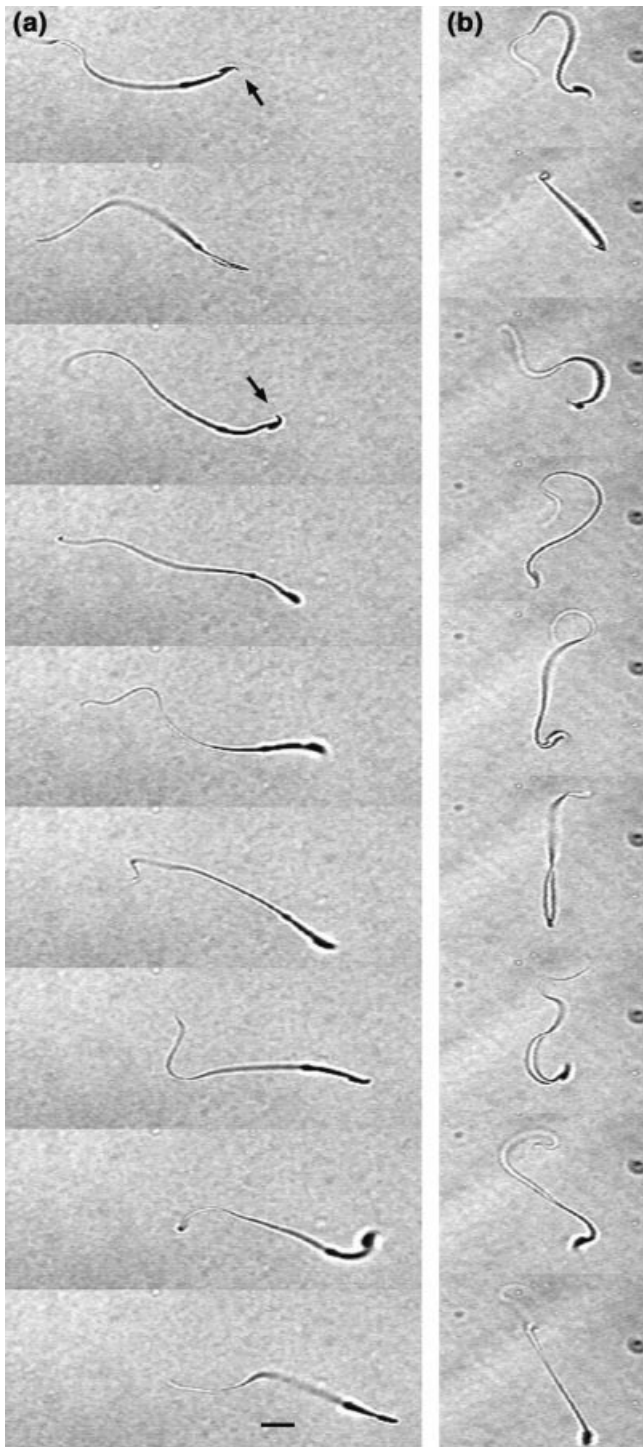


Fig. 2. Phase-contrast video images of (a) activated and (b) hyperactivated mouse spermatozoa. Consecutive images were collected at 30 Hz and each series begins at the top of the plate. Rotation of the spermatozoa about its long axis (rolling) can be seen by noting the position of the hook on the head (arrows). Scale bar represents 10 μm .

(Suarez, 1988). After 2–3 h of incubation under capacitating conditions, rolling increases and they swim in a helical pattern. Rolling is diminished once again at hyperactivation, so the spermatozoa trace out small circles (Suarez, 1988). Rolling that occurs once per beat cycle produces a helical path, whereas rolling that occurs intermittently causes a change in the direction of swimming. Thus, rolling has the potential to provide a chemotactic response.

A second factor that affects swimming patterns is the thickness of the outer dense fibres in the flagellum (Fig. 3) (Phillips, 1972). Species showing a small radius of curvature in flagellar bends (mice, humans, rabbits, opossums) tend to have smaller outer dense fibres than do species showing a large radius of curvature in the flagellar bend (rats, Chinese hamsters).

Two additional factors that affect flagellar movement patterns are apparently unique to marsupial spermatozoa. Spermatozoa of American opossums (*Monodelphis domestica*, *Didelphis virginiana*) are bound together by the heads, forming a biflagellate organism; the pairs separate before fertilization. Spermatozoa of *Monodelphis domestica*, obtained from the distal caudal epididymis, were paired and swam progressively, with flagella beating synchronously, until a few hours of incubation in fertilization medium (Moore and Taggart, 1993). Subsequently, some pairs began to beat asynchronously until the spermatozoa pulled apart. At this stage, some of the single spermatozoa showed a ‘whiplash’ flagellar movement resembling hyperactivation. Only unpaired spermatozoa bound to the zona pellucida. In some Australian marsupials, the joint between the head and flagellum is modified in the oviduct before fertilization, such that the longitudinal axis of the head shifts from being in alignment with the flagellum, to orienting perpendicularly to the flagellum (Bedford and Breed, 1994).

Because of species differences in movement patterns, objective evaluation of hyperactivation using computer-assisted sperm analysis (CASA) must be determined for each species. A common procedure is to incubate spermatozoa under capacitating conditions and compare swimming patterns with spermatozoa that have not been exposed to capacitating conditions. A particular subset of movement patterns observed to increase under capacitating conditions is categorized as hyperactivated. The incidence of hyperactivation is assayed by setting threshold values for movement measurement variables that distinguish this subgroup from others (mouse: Neill and Olds-Clarke, 1987; human: Mortimer and Swan, 1995; ram: Mortimer and Maxwell, 1999; rat: Cancel *et al.*, 2000). It should be noted that most CASA systems operate by measuring the movements of the head, which measures flagellar movement only indirectly.

A physiological signal for hyperactivation?

Hyperactivation must be switched on at the right place and time to achieve fertilization. Thus, it is likely that there are

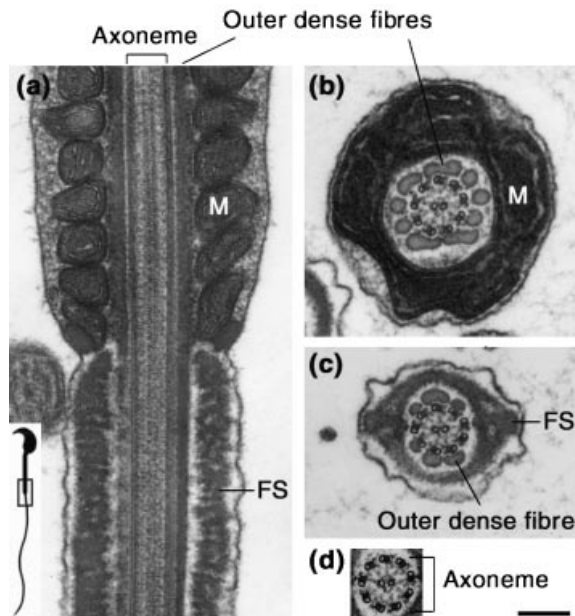


Fig. 3. (a) Longitudinal section of the junction of the midpiece and principal piece of a mouse spermatozoon (boxed area shown in inset). The axoneme, consisting of microtubules, dynein ATPase and associated proteins, is in the core of the flagellum. It is surrounded by outer dense fibres and mitochondria (M) in the midpiece, and by fibrous sheath (FS) in the principal piece. (b,c) Cross-sections of the midpiece and principal piece, respectively. (d) Cross-section of the axoneme alone, showing the typical '9+2' pattern, with a central pair of microtubules surrounded by nine sets of doublets. Scale bar represents 200 nm.

one or more specific signals within the female reproductive tract for regulating hyperactivation. There is evidence that various components of the female reproductive tract may serve as physiological stimuli of hyperactivation. Hormones, ions and secretions in the oviductal luminal fluid vary during the oestrous cycle (Nichol *et al.*, 1992). When the oocyte enters the oviduct, it usually brings with it cumulus oophorus and follicular fluid, which influence sperm motility.

Human follicular fluid has a dose-dependent effect on flagellar beat frequency and hyperactivation (Yao *et al.*, 2000). It was suggested that progesterone is the active factor, because it enhances hyperactivation (Sueldo *et al.*, 1993). However, progesterone did not stimulate hyperactivation in at least one study (Kay *et al.*, 1994) and it seems unlikely that progesterone alone induces hyperactivation, because progesterone receptors are confined to the human sperm head (Blackmore and Lattanzio, 1991).

Cumulus cells that enter the ampulla with the oocyte may also provide signals for hyperactivation. Culture medium exposed to cumulus oophorus cells increases curvilinear velocity and amplitude of lateral head displacement of human spermatozoa (Fetterolf *et al.*, 1994).

Despite evidence from experiments *in vitro* for a role of

follicular fluid and cumulus in initiating hyperactivation, evidence *in vivo* argues that these factors alone cannot be responsible for initiating hyperactivation. Hyperactivated spermatozoa have been detected in the oviduct before ovulation in rabbits (Cooper *et al.*, 1979) and mice (Suarez and Osman, 1987). Although follicular fluid was found to reach the oviductal ampulla at ovulation in pigs, only < 1% of the fluid was retained in the ampullary lumen (Hansen *et al.*, 1991); an even smaller amount would reach the spermatozoa stored in the reservoir in the caudal isthmus. Alternatively, the follicle may communicate with the spermatozoa in the isthmus while still in the ovary. There is anatomical evidence for a vascular counter-current transfer of follicular hormones from veins leaving the ovary to arteries supplying the wall of the isthmus (Hunter *et al.*, 1983).

Although follicular fluid and cumulus components have been observed to induce hyperactivation, no specific signals have been identified unequivocally.

Mechanisms regulating hyperactivation

A number of physiological factors, such as Ca^{2+} , cAMP, bicarbonate and metabolic substrates, are essential for the initiation and maintenance of hyperactivated motility *in vitro*.

Ca^{2+} plays a major role in regulating hyperactivated motility. Extracellular Ca^{2+} is required to maintain hyperactivation in hamster spermatozoa *in vitro* (Yanagimachi, 1994), and treatment of mouse spermatozoa with Ca^{2+} ionophore A23187 rapidly induces hyperactivated motility (Suarez *et al.*, 1987). Cytoplasmic Ca^{2+} concentrations within the flagella of hyperactivated hamster spermatozoa are higher than those in activated spermatozoa (Suarez *et al.*, 1993). A demembrated sperm model (treating spermatozoa with Triton-X100 to disrupt plasma membrane and mitochondrial membranes, essentially leaving only the axoneme and cytoskeletal support (Fig. 3)) has been used to study hyperactivated motility. When rat and bull spermatozoa were treated with Triton-X100, they began swimming if ATP and cAMP were added and became hyperactivated if Ca^{2+} was added (Lindemann and Goltz, 1988; Lindemann *et al.*, 1991). These studies indicate that the crucial site for the action of Ca^{2+} is the axoneme (Fig. 3). However, the mechanism by which Ca^{2+} reaches the axoneme in the core of the flagellum to switch on hyperactivation in intact spermatozoa is still unknown.

Procaine-treated guinea-pig spermatozoa undergo hyperactivation in Ca^{2+} -deficient medium (Mujica *et al.*, 1994), indicating that there may be an intracellular Ca^{2+} store in the flagellum to provide increased Ca^{2+} to the axoneme. Inositol 1,4,5-trisphosphate (IP_3) releases Ca^{2+} from some stores, and IP_3 receptors have been localized by antibodies to the base of the flagellum (Walensky and Snyder, 1995). Heterotrimeric G proteins of the $\text{G}_{q/11}$ family selectively activate the β isoforms of the phosphatidylinositide-specific phospholipase C to produce IP_3 . Both $\text{G}\alpha_{q/11}$ and

phospholipase C- β have been localized along the midpiece of rat sperm flagella (Walensky and Snyder, 1995).

In addition to the IP₃ receptor-gated Ca²⁺ store, extracellular Ca²⁺ may be required to maintain hyperactivation for long periods by replenishing the intracellular store and maintaining high cytoplasmic Ca²⁺. When extracellular Ca²⁺ was unavailable, hyperactivated hamster spermatozoa reverted to activated motility (Yanagimachi, 1994). Cyclic nucleotide-gated channels have been localized to bull sperm flagella (Wiesner *et al.*, 1998); therefore, activation of cyclic nucleotide-gated channels by cAMP/cGMP may be one of the mechanisms providing Ca²⁺ entry.

Increased intracellular cAMP has been associated with the expression of hyperactivated motility (White and Aitken, 1989). Aoki *et al.* (1999) proposed that cAMP might be involved in the regulation of flagellar waveform by maintaining Ca²⁺ at an appropriate concentration. Ca²⁺ and cAMP may also modulate protein phosphorylation (Tash, 1989). Increased protein tyrosine phosphorylation in flagella is associated with hyperactivated motility in hamster (Si and Okuno, 1999) and monkey spermatozoa (Mahony and Gwathmey, 1999). Tyrosine phosphorylation has also been associated with heat-induced hyperactivation in human spermatozoa (Chan *et al.*, 1998). Tyrosine phosphorylation, dephosphorylation and rephosphorylation of an 80 kDa protein in hamster sperm flagella are tightly associated with the acquisition, loss and reacquisition of temperature-dependent hyperactivation (Si, 1999). As dephosphorylation has been observed, phosphatases are also presumed to be involved in the regulation of hyperactivation.

Bicarbonate supports hyperactivation in mouse spermatozoa (Neill and Olds-Clarke, 1987), possibly by modulating the activity of adenylyl cyclase in the flagellum (Okamura *et al.*, 1985). A soluble form of mammalian adenylyl cyclase has been purified and cloned recently, and can be stimulated directly by bicarbonate (Chen *et al.*, 2000). It will be informative to determine whether bicarbonate regulates sperm motility through direct activation of the soluble adenylyl cyclase.

Although cAMP and Ca²⁺ regulate hyperactivated motility through different pathways, they also modulate the actions of each other. Ca²⁺ causes marked increases in cAMP and activates the Mg²⁺-dependent adenylyl cyclase of guinea-pig spermatozoa (Hyne and Garbers, 1979). In spermatozoa treated with Triton-X100 to remove the plasma and mitochondrial membranes, the response to Ca²⁺ appears to require cAMP priming (Lindemann *et al.*, 1991). However, Mujica *et al.* (1994) showed that cAMP was present at high concentrations in guinea-pig spermatozoa independent of hyperactivation. It appears that cAMP is required but is not sufficient for hyperactivation.

It should be stressed that Ca²⁺ is responsible for increasing flagellar asymmetry, which is characteristic of hyperactivated motility, whereas cAMP is a major factor in the initiation and maintenance of flagellar beats. In a study using sea urchin spermatozoa as a model, selective regulation of the activity of

a group of dynein arms by Ca²⁺ was demonstrated to be the basis for the changes in flagellar bending patterns brought about by high Ca²⁺ concentrations (Bannai *et al.*, 2000). As calmodulin is known to be responsible for increasing the asymmetry of the flagellar waveform in the presence of Ca²⁺ above 10⁻⁶ mol l⁻¹ (Brokaw, 1991), it is possible that a Ca²⁺-calmodulin complex regulates the activity of dynein through protein phosphorylation.

In summary, it appears that Ca²⁺ is the most important factor regulating hyperactivation, and that cAMP is required but is not sufficient for expression of hyperactivation. A hypothetical model for regulation of hyperactivation is proposed (Fig. 4).

Hyperactivation and chemotaxis/chemokinesis

Chemotaxis has been well documented in invertebrates, in which very low concentrations of peptides, such as speract secreted by sea urchin eggs, attract spermatozoa in a species-specific manner. The peptides facilitate fertilization by binding to sperm receptors and thereby stimulating motility and respiration. Cook *et al.* (1994) demonstrated that intracellular Ca²⁺ mediates chemotaxis in sea urchin spermatozoa, whereby increases in intracellular Ca²⁺ enhance flagellar bend asymmetry and swimming path curvature. In a similar way to chemotactic responses of sea urchin spermatozoa, hyperactivated mammalian spermatozoa display increased flagellar bend asymmetry and change direction frequently. These movements could allow spermatozoa to reach the oocyte. Analogous ligand-receptor interactions in vertebrates have not been characterized, although human spermatozoa are attracted by follicular fluid *in vitro* (Ralt *et al.*, 1994).

Odorant-like molecules could function as chemo-attractants to spermatozoa to guide them to the oocyte. Antibodies to a conserved region of odorant receptors specifically labelled mature dog spermatozoa (Vanderhaeghen *et al.*, 1993) and the base of the flagellum and the proximal midpiece of mature rat spermatozoa (Walensky *et al.*, 1995). These antibodies also detected odorant receptors in western blots of protein extracts of hamster and human spermatozoa (Walensky *et al.*, 1995). In permeabilized preparations of olfactory cilia, odorants were found to stimulate cAMP and IP₃ production (Pace *et al.*, 1985). In the base of the sperm flagellum, putative chemotactic ligand-receptor interactions could produce an IP₃-induced release of Ca²⁺, which may, in turn, contribute to high intracellular Ca²⁺ concentrations where the flagellar bend initiates and to a subsequent change in motility.

Speract activates a transmembrane guanylyl cyclase-receptor complex and apparently utilizes cGMP to activate sea urchin sperm K⁺ channels; speract also causes a transient increase in cAMP and Ca²⁺ (Cook and Babcock, 1993). Cyclic nucleotide-gated channels have been localized along the midpiece and principal piece of bull spermatozoa, and activation of these channels increases intracellular Ca²⁺ (Wiesner *et al.*, 1998). The localization of cyclic nucleotide-gated channels along the flagellum

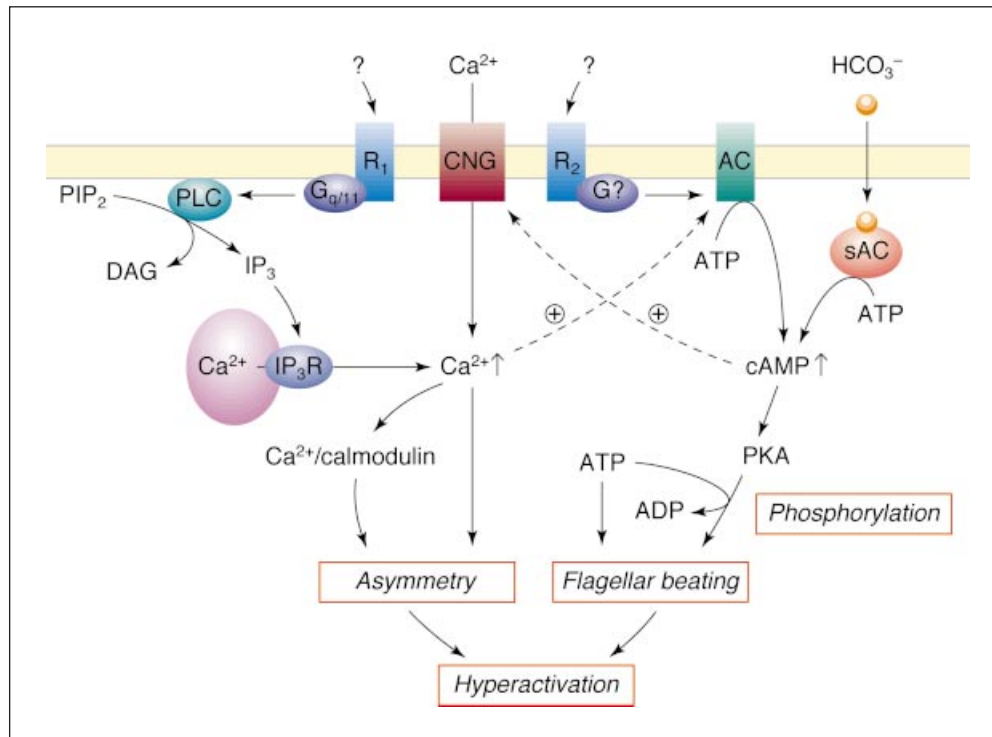


Fig. 4. A hypothetical model for mechanisms regulating hyperactivation. Upon signal stimulation, pathways leading to increases in Ca^{2+} and cAMP are turned on. Unknown physiological signals activate phospholipase C (PLC) through a heterotrimeric G protein ($G_{q/11}$)-coupled receptor (R_1) and produce IP_3 . Binding of IP_3 to IP_3 receptors (IP_3R) causes an increase in cytoplasmic Ca^{2+} . Activation of the membrane-associated adenylyl cyclase (AC) through high cytoplasmic Ca^{2+} and possibly G proteins ($G?$) and membrane potential increases intracellular cAMP. Bicarbonate may also cause an increase in cAMP by activating the soluble form of adenylyl cyclase (sAC) directly. The increased cAMP can bind to cyclic nucleotide-gated channels (CNG) to induce Ca^{2+} influx. Increased cAMP activates protein kinase A (PKA) to phosphorylate axonemal or fibrous sheath proteins and results in flagellar beating. High cytoplasmic Ca^{2+} and Ca^{2+} -calmodulin complex are responsible for asymmetrical bending of flagella that is characteristic of hyperactivation.

implies that flagellar bending waves could be regulated in a chemotactic-like manner.

Hyperactivation and capacitation

The physiological changes that allow spermatozoa to undergo the acrosome reaction are collectively called 'capacitation' (Yanagimachi, 1994). Although hyperactivation usually occurs at some point during capacitation *in vitro*, the pathways leading to hyperactivation and capacitation are not coupled completely. Boatman and Robbins (1991) found that bicarbonate is essential for both capacitation and hyperactivation of hamster spermatozoa *in vitro*, but hyperactivation required higher concentrations of bicarbonate than did capacitation. Hyperactivation can proceed independently of capacitation in mouse and hamster spermatozoa. Spermatozoa from $t^{w32/+}$ mouse show hyperactivated motility prematurely *in vitro*, whereas capacitation occurs on schedule (Neill and Olds-Clarke, 1987; Olds-Clarke, 1989). In hamster spermatozoa,

medium with certain energy substrates (BSA, adrenaline, hypotaurine and penicillamine) allows full development of hyperactivation, which precedes capacitation by ≥ 1 h (DeMott *et al.*, 1995). Procaine can initiate hyperactivation rapidly in uncapacitated guinea-pig spermatozoa (Mujica *et al.* 1994). Thus, hyperactivation and capacitation should be considered as independent events.

Conclusions

Hyperactivated motility appears to be critical to the process of fertilization *in vivo*, allowing spermatozoa to reach the oocyte through the narrow, mucus-filled, labyrinthine lumen of the oviduct and assisting spermatozoa in penetrating the cumulus oophorus and the zona pellucida. The signal initiating hyperactivation in the oviduct has not been identified; however, Ca^{2+} is a critical element in the signalling pathway to convert symmetrical bending to the asymmetrical bending that is characteristic of hyperactivation. This vigorous movement also requires ATP and

cAMP. Determining how hyperactivation is initiated in the oviduct would provide valuable information in the diagnosis and treatment of infertility or in the development of new methods of contraception that prevent spermatozoa from reaching the oocyte.

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