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Author: Inaba, Kazuo

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[REVIEW]

Molecular Architecture of the Sperm Flagella: Molecules for Motility and Signaling

Kazuo Inaba*

Asamushi Marine Biological Station, Graduate School of Science, Tohoku University, Sakamoto 9, Asamushi, Aomori, Aomori 039-3501, Japan

ABSTRACT—Sperm motility is generated by a highly organized, microtubule-based structure, called the axoneme, which is constructed from approximately 250 proteins. Recent studies have revealed the molecular structures and functions of a number of axonemal components, including the motor molecules, the dyneins, and regulatory substructures, such as radial spoke, central pair, and other accessory structures. The force for flagellar movement is exerted by the sliding of outer-doublet microtubules driven by the molecular motors, the dyneins. Dynein activity is regulated by the radial spoke/central pair apparatus through protein phosphorylation, resulting in flagellar bend propagation. Prior to fertilization, sperm exhibit dramatic motility changes, such as initiation and activation of motility and chemotaxis toward the egg. These changes are triggered by changes in the extracellular ionic environment and substances released from the female reproductive tract or egg. After reception of these extracellular signals by specific ion channels or receptors in the sperm cells, intracellular signals are switched on through tyrosine protein phosphorylation, Ca^{2+}, and cyclic nucleotide-dependent pathways. All these signaling molecules are closely arranged in each sperm flagellum, leading to efficient activation of motility.

Key words: sperm, motility, flagella, axoneme, dynein

INTRODUCTION

As the tails of sperm, flagella comprise the motile apparatus necessary for the movement and penetration of sperm into the egg at fertilization (Fig. 1A, B). They show oscillatory movements at high speed. The motility is generated by the internal cytoskeletal structure called the axoneme, which is a highly organized microtubule-based structure that has been well conserved through evolution (Fig. 1C). The axoneme also serves as the motility or sensory apparatus of cilia on the trachea, oviduct, and sensory organs. Mammalian sperm flagella are divided into two parts, the midpiece and the principal piece, and accessory structures are present between the axonemes and the plasma membrane (Fig. 1B). In the midpiece of the flagellum, the axonemes are surrounded by outer dense fibers (ODF) and mitochondria, while a fibrous sheath (FS) surrounds the axoneme in the principal piece (Fig. 1D–F) (for review, see Baccetti and Afzelius, 1976).

* Corresponding author: Tel. +81-17-752-3394;
FAX. +81-17-752-2765.
E-mail: inaba@biology.tohoku.ac.jp

Immediately after spermiogenesis, sperm cells show no or little motility. Prior to fertilization, however, sperm motility changes dramatically. For example, sperm from animals with external fertilization show activation of motility by changes in the extracellular ionic environment or by substances released from the egg. Sperm from animals with internal fertilization show activation by factors in the female reproductive tract or substances from the egg. All of these processes are triggered by reception of the respective stimuli. The motility machinery, the axoneme, is finally activated as the end-stage of the intracellular signaling pathway (Morisawa, 1994; Darszon *et al*., 2001). The molecules involved in this signaling pathway appear to be highly organized, as are the axonemes, to facilitate such a prompt response.

The structure and function of axonemes have been widely studied in *Chlamydomonas* using several mutants with motility defects (reviewed in Mitchell, 2000). Recent studies have explored the molecular architecture of the axoneme in detail. In addition, the signaling mechanism for modulation of sperm motility has been clarified by the recent identification of several molecules, including receptors,

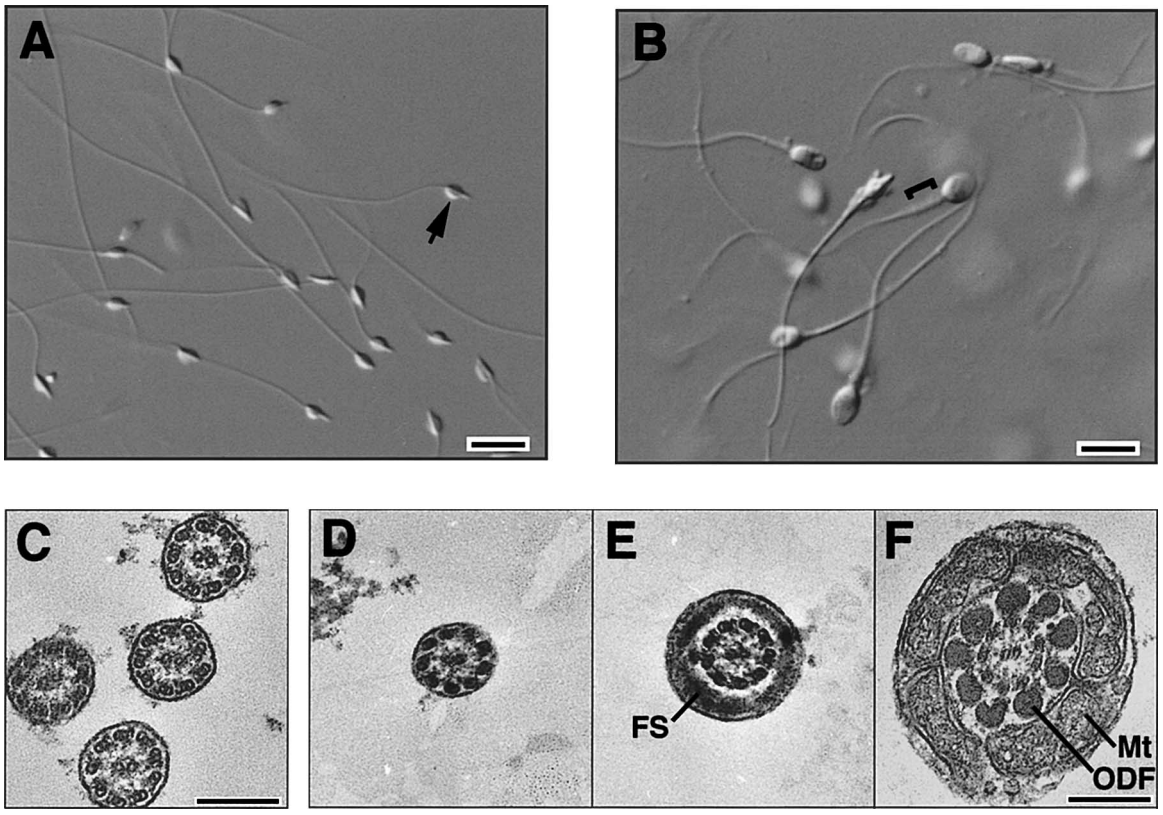

Fig. 1. Morphology of ascidian and mammalian sperm. A, DIC image of sperm from the ascidian *Ciona intestinalis*. The arrow shows a mitochondrion attached to the side of a nucleus. Bar, 10 μm. *B*, DIC image of human sperm. The region of the midpiece is indicated. The rest of the flagellum is the principal piece, except for the small portion at the tip called the endpiece. Bar, 10 μm. *C*, Electron microscopic image of the cross-section of *Ciona* flagellum. The axonemal 9+2 structure is surrounded by plasma membrane. Bar, 200 nm. *D–F*, Electron microscopic images of several portions of human sperm flagella. *D*, endpiece; *E*, principal piece; *F*; midpiece. The endpiece has no accessory structure between the axoneme and the plasma membrane. A fibrous sheath (FS) is present between them in the principal piece. In the midpiece, the axoneme is surrounded by nine rows of outer dense fiber (ODF), which are further wrapped by mitochondria (Mt). The scale bar in D–F indicates 200 nm.

channels, and signal molecules, involved in the activation of motility. Functional genomics and proteomics are expected to provide new ways to carry out extensive characterization of the components of flagella. In this review, molecules constructing sperm flagella and essential for the regulation of sperm motility are described. The detailed structure and function of each protein molecule have been described in previous review articles (Gibbons, 1981; Kamiya, 2002; Morisawa, 1994; Ho and Suarez, 2001; Darszon *et al*., 2001; Garbers, 1989).

I. Axonemes: The motility apparatus of sperm flagella

The 9 + 2 structure and molecular composition of the axoneme are well conserved among eukaryotic cilia and flagella from protozoans to human (Fig. 2). The doublet microtubules, numbered from 1 to 9, are composed of a complete A-tubule and an incomplete B-tubule. The central microtubules are named C1 and C2. Several structures are bound to these microtubules and comprise a highly organized protein network (Fig. 2). Axonemes are composed of approximately 250 proteins. Extensive studies of the molecular composition of axonemes have been carried out with the green alga *Chlamydomonas*. However, more than half of the protein components remain to be characterized. Knowledge of *Chlamydomonas* axonemes can be applied to sperm axonemes, but there are some differences in the composition and molecular structure between *Chlamydomonas* and metazoan sperm. Recent molecular studies to identify axonemal components revealed that the axoneme is a sophisticated structure with a cytoskeleton, protein motors, molecular chaperones, Ca^{2+}-binding proteins and protein kinases / phosphatases. Some axonemal proteins possess motifs that are potentially essential for protein-protein interactions, facilitating the assembly of such a highly organized structure. The axonemes are generated from the basal body where a set of transient structures are connected to the axoneme. Axonemal components are integrated through a microtubule-dependent transport system, called intraflagellar transport (IFT). The basal body and its associated structure, the composition of IFT particles, and the mechanism of IFT have been reviewed elsewhere (Marshall and Rosenbaum, 2000; Rosenbaum and Witman, 2002).

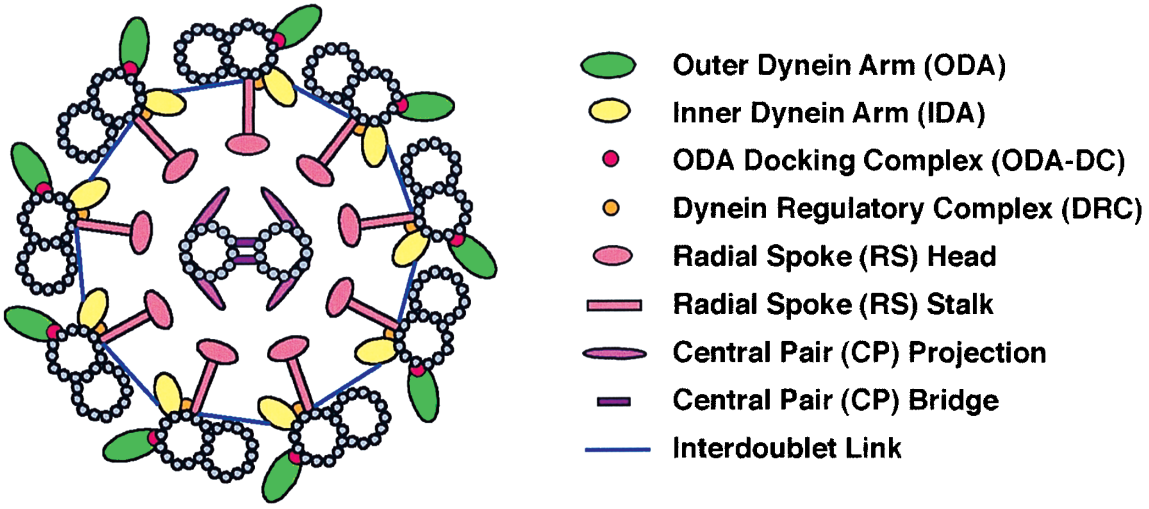

Fig. 2. Substructures in the axoneme. Axonemes are constructed from nine doublet microtubules and two singlet microtubules as shown by gray rings. Each doublet microtubule is a unit for sliding, with dynein arms, the dynein docking complex, radial spokes, and the dynein regulatory complex. Nine doublet microtubules are connected with each other by interdoublet links. The singlet microtubules at the center are joined by the central pair bridge. Each microtubule possesses two central pair projections. The molecular composition of each substructure is detailed in the text.

Dynein arms

Dyneins are microtubule-dependent force-generating ATPases. The dyneins involved in the motility of eukaryotic cilia and flagella are classified as axonemal dyneins. Dyneins are observed as a pair of projecting "arms" on doublet microtubules designated the outer and inner arms. The structure of outer arm dynein has been studied extensively. In metazoan sperm, it is comprised of two heavy chains (~500 kDa) (three in *Chlamydomonas*), three to five intermediate chains (120–60 kDa) (two in *Chlamydomonas*), and six (eight in *Chlamydomonas*) light chains (30–8 kDa).

Heavy chains: Dynein heavy chains are huge proteins with a molecular mass of ~500 kDa. The outer arm dynein contains two heavy chains, α and β, which are related phylogenically to γ and α/β heavy chains of *Chlamydomonas* outer arm dynein, respectively. Each heavy chain appears to play a distinct function in microtubule-sliding. They contains four P-loop ATP-binding motifs (Ogawa, 1991; Gibbons *et al*., 1991). Recent studies have shown that dynein heavy chains are members of the AAA superfamily and have six AAA domains (Mocz and Gibbons, 2001; King, 2000b). Each AAA motif is considered to form a globular subdomain. The coiled-coil domain present between the fourth and fifth AAA motif forms a small stem that is essential for ATP-sensitive binding to the adjacent B-tubule (King 2000a; Gee *et al*., 1997). The interzone between each AAA domain appears to be important for the conformational change during mechanochemical cycles (Inaba and Mohri, 1989; Inaba, 2000), which presumably exerts the power stroke (Burgess *et al*., 2003).

Intermediate chains: *Chlamydomonas* outer arm dynein contains two intermediate chains, IC78 and IC69, of which clear homologs are present in sperm flagella (Ogawa *et al*., 1995). Both possess WD-repeats, which are involved in protein-protein interaction and possibly play a key role in assembly and binding of dynein on the A-tubule. Sperm flagella contain a unique intermediate chain with thioredoxin and nucleoside diphosphate kinase (TNDK-IC) motifs (Ogawa *et al*., 1996; Padma *et al*., 2001). In sea urchin sperm flagella, this intermediate chain shows a high molecular mass (~120 kDa), but the size of TNDK-IC has become smaller during evolution (Padma *et al*., 2001). In *Ciona*, salmonid fish and mollusca, outer arm dynein contains two or three other intermediate chains (Ogawa *et al*., 1996; Padma *et al*., 2001), but these remains uncharacterized.

Light chains: There are six or eight distinct proteins identified as outer arm dynein light chains in metazoan sperm flagella and *Chlamydomonas* flagella, respectively (Inaba *et al*., 1998, 1999; King, 2000). Two of these molecules show homology to t-complex testis-expressed proteins (Tctex1 and Tctex2), which are involved in transmission ratio distortion in mouse (Olds-Clarke, 1997). The Tctex2-related dynein light chain is phosphorylated at activation of sperm motility in a cAMP-dependent manner and may play a key role in the activation of outer arm dynein (Inaba *et al*., 1999). The other molecules include a leucine-rich repeat (LRR) protein and two isoforms homologous to highly conserved *Chlamydomonas* 8-kDa light chains. Tctex1 light chain is not present in the outer arm dynein of *Chlamydomonas*. One light chain (LC5) remains to be identified. A small protein with homology to *Drosophila* roadblock has been identified in *Chlamydomonas* (King, 2002) and it could be a possible candidate for sperm LC5. Although *Chlamydomonas* outer arm dynein contains Ca^{2+}-binding light chain but no Ca^{2+}-binding proteins have been found in metazoan outer arm

dynein. Calmodulin (CaM) was found to be associated with outer arm dynein in sea urchin and mammalian sperm flagella (Tash *et al*., 1988). A 25-kDa protein with sequence similarity with calcineurin B subunit has recently been identified in association with *Ciona* outer arm dynein (Padma and Inaba, manuscript in preparation).

Outer arm docking complex: The factor for assembly of outer arm dynein to the doublet microtubules at regular intervals (24 nm) was first identified in *Chlamydomonas* (Takada and Kamiya, 1994) and was designated outer dynein arm docking complex (ODA-DC). This molecule sediments at 7 S on sucrose density gradient centrifugation and is composed of three polypeptides. Both *Chlamydomonas* DC1 and DC2 are coiled coil proteins that may be involved in scaling on microtubules for binding of outer arm dynein at regular intervals (Koutoulis *et al*., 1997; Takada *et al*., 2002). DC3 has four EF-hand motifs that possibly bind Ca^{2+} and have some roles in the regulation of outer arm dynein (Casey *et al*., 2003). We have recently identified a DC2 homolog (CiAx p66.0) in *Ciona* sperm flagella, but part of this protein was isolated bound to outer arm dynein (Ushimaru *et al*., manuscript in preparation; Accession number, AB083180). However, proteins with significant similarity to DC1 and DC3 could not been identified in metazoans. Although similar ODA-DC may be present in metazoan sperm, its composition and binding properties to doublet microtubules may be different between *Chlamydomonas* and metazoan sperm flagella.

Subunits of inner arm dynein: Inner arm dyneins are more complex than outer arm dyneins, and include multiple molecular species with more heavy chains. Inner arm dyneins in *Chlamydomonas* have been shown to contain seven species (a-f) (Kamiya, 2002). The inner arms of eel sperm axonemes are morphologically divided into three species (Woolley, 1997), similar to those in *Chlamydomonas* (Goodenough and Heuser, 1985). *Chlamydomonas* f inner arm dynein (also called I1) contains intermediate chains IC140, IC138, IC97, the 14-kDa Tctex1 light chain, and an 8-kDa light chain (Kamiya, 2002). IC138 is regulated by phosphorylation / dephosphorylation through a kinase/phosphatase system present in the radial spoke and central pair in response to changes in motility (Habermacher and Sale, 1997; King and Dutcher, 1997). A homolog of IC140 (Ci-IC116) has recently been isolated from sperm flagella of the ascidian *Ciona intestinalis*. Similarly to *Chlamydomonas* IC140 (Yang and Sale, 1998; Perrone *et al*., 1998), *Ciona* IC116 contains WD repeats, suggesting that it is involved in the assembly or anchoring of inner arm dyneins to the microtubule. However, Ci-IC116 appears to be dephosphorylated at activation of motility, suggesting a regulatory role of this protein (Inaba *et al*., 2002). *Chlamydomonas* inner arm dyneins contain p28, actin, centrin, and Tctex1 and a few unidentified proteins as light chains (Kamiya, 2002). A sea urchin homolog of *Chlamydomonas* p28 (p33) was reported to be present in axonemes as a putative inner arm dynein light chain (Gingras *et al*., 1996), but its detailed localization has not been elucidated. Actin is found in inner arm dyneins in association with a p28 homolog in fish sperm (King *et al*., 1997), as in the case of *Chlamydomonas* (Piperno *et al*., 1990; Kato-Minoura *et al*., 1997). A set of proteins form a complex at the junction between radial spokes and inner arm dynein, called the dynein regulatory complex (DRC) (Piperno *et al*., 1994 Gardner *et al*., 1994). Seven polypeptides (29-192 kDa) were proposed as DRC components in *Chlamydomonas* but neither molecular characterization nor the presence of homologs in metazoan sperm have been reported.

Radial spoke and central pair: Several lines of evidence support the idea that the central pair determines the plane of flagellar bending by sending signals to radial spokes (Smith and Lefebvr, 1997b; Nakano *et al*., 2003). It was recently suggested that the C1 microtubule is oriented toward the position of active sliding (Wargo and Smith, 2003). Radial spokes regulate inner arm dynein through protein phosphorylation / dephosphorylation (Porter and Sale, 2000). In fact, among the 22 polypeptides identified to date as radial spoke proteins in *Chlamydomonas* flagella, 97-kDa RSP3 (radial spoke protein 3) has been shown to be located at the base of the radial spoke stalk and possesses an AKAP domain that anchors a cAMP-dependent protein kinase (A-kinase) (Gaillard *et al*., 2001). RSP3 of *Ciona* axoneme also has an AKAP domain but its entire length is much shorter than that of *Chlamydomonas* RSP3 (Padma *et al*., 2003). CaM is also a component of radial spokes and may play a role in Ca^{2+}-dependent changes in the flagellar waveform (Yang *et al*., 2000). Radial spokes also contain RSP4/6 in the spoke head. RSP4/6 is also a component of sea urchin sperm radial spokes (Gingras *et al*., 1998). Recently, a novel leucine-rich repeat (LRR) component of the radial spoke head (LRR37) has been identified (Padma *et al*., 2003). In view of the possible function of LRR in protein-protein interaction, it might be involved in the interaction with other components of the radial spoke or with components of central pair projection.

Some proteins constituting central pair projections have been identified using *Chlamydomonas* mutants (Mitchell, 2000). Approximately 23 polypeptides (14-360 kDa) comprise the structure associated with central pair microtubules (Adams *et al*., 1981; Dutcher *et al*., 1984). Similarly to the radial spokes, the central pair also contains an AKAP (AKAP240) (Gaillard *et al*., 2001). Genes for PF6 (alanine/proline-rich protein; Rupp *et al*., 2001), PF16 (armadillo repeat protein; Smith and Lefebvre, 1996), and PF20 (WD repeat protein; Smith and Lefebvre, 1997a) have been cloned and well characterized in *Chlamydomonas*. Orthologs of PF16 (Spag6) and PF20 are associated with each other in mammalian sperm (Sapiro *et al*., 2002; Zhang *et al*., 2002). However, there are no orthologs with significant homology to PF6 over the entire length of the sequence

in metazoan sperm. A kinesin-related protein, KLP1, and a 110-kDa protein that is immunologically related to a kinesin, were identified as components of the central apparatus in *Chlamydomonas* (Bernstein *et al*., 1994; Johnson *et al*., 1994). The precise function and the presence of the kinesin-related protein in the central pair of sperm flagella are unknown, although subunits of kinesin II appear to be localized at the midpiece of sea urchin sperm and seem to be involved in intraflagellar transport (Henson *et al*., 1997).

Other proteins in relation to axonemes: Seven members of the tubulin superfamily, the α- to ε-tubulins, have been identified (Dutcher, 2001). The γ- δ-, ε-, ζ-, and η-tubulins are localized at the basal body and are probably involved in construction of the centriole or formation of the axoneme. In mouse sperm, δ-tubulin is localized in several parts of the sperm cells, including the principal piece of the flagellum (Smrzka *et al*., 2000), suggesting a distinct function of this tubulin in the axonemal architecture. The α- and β-tubulins undergo posttranslational modification, such as acetylation, palmitoylation, tyrosine phosphorylation, polyglutamylation and polyglycylation. These modifications play roles in microtubule functions, such as microtubule stability and the interaction with associated proteins (Huitorel *et al*., 1999). Some of these modifications, such as polyglutamylation, apparently participate in axonemal motility (Gagnon *et al*., 1996).

Flagellar ribbons are Sarkosyl-resistant structures that are localized along the A-tubule and may play a role in the three-dimensional organization of the axoneme (Norrander *et al*., 1996). Ribbons are composed of three fibrous proteins called tektins (53, 51, and 47 kDa), along with 83-kDa, 77-kDa, and several lower molecular mass proteins in sea urchin sperm flagella (Hinchcliffe and Linck, 1998). The ribbons presumably connect with interdoublet links and are involved in the architecture of the ninefold axonemal remnant after solubilization of the outer doublet microtubules (Stephens *et al*., 1989). A cognate of the heat shock protein HSP70 also seems to be associated with this remnant (Stephens and Lemieux, 1999), but the function of this cognate molecule with regard to the role of HSP70 as a molecular chaperone remains to be determined. Recently, HSP40 was identified as an axonemal component of the Ciona sperm axoneme (Padma *et al*., 2003). HSP40 itself functions as a molecular chaperone but is known to also act as a co-chaperone of HSP70. Preliminary experiments showed that HSP40, like HSP70, is resistant to extraction by low ionic strength solution, suggesting the possibility that the HSP70 cognate and HSP40 form a complex and function in the architecture of doublet microtubules.

Interdoublet links, or "nexin" linkages, are the structures that link the outer doublet microtubules. From the results obtained by observation of the outer arm-less axonemes from eel sperm, however, it has been proposed that the interdoublet link is the major part of the DRC (Wooley, 1997). Using selective extraction, the interdoublet links were shown to be comprised of certain polypeptides (Stephens, 1970). Recently, a strong candidate as a component of interdoublet links, Rib72, with DM10 repeats and EF-hands, was cloned from *Chlamydomonas* as a ribbon component (Ikeda *et al*., 2003). This protein has since been shown to be the p72 regulatory subunit of Ca^{2+}-regulated nucleoside-diphosphate kinase (Patel-King *et al*., 2002). A homolog of this protein is present in *Ciona* and human, suggesting that it is also a component of sperm flagella.

Protein kinases / phosphatases and Ca^{2+}-binding proteins associated with axonemes: Protein phosphorylation plays essential roles in the regulation of axonemal movement (see below). The catalytic subunit of cAMP-dependent protein kinase in sperm flagella has a unique testis-specific structure. In salmonid fish (Itoh *et al*., 2003) and mammals (San Agustin *et al*., 1998; Agustin *et al*., 2000), the testis-specific PKA catalytic subunit has a lower molecular weight with a shorter N-terminus sequence, which may be involved in anchoring to the microtubules. PKA is located in the vicinity of outer arm dynein, which is consistent with the prompt phosphorylation of Tctex2-related dynein light chain observed on activation of motility (Inaba *et al*., 1999; Itoh *et al*., 2003). The t-complex responder has been shown to be a protein kinase (Smok) with similarities to members of the MARK Ser/Thr protein kinase family (Herrmann *et al*., 1999). However the substrate proteins of Smok have not been identified in sperm.

In salmonid fish sperm, proteasomes are also located near the outer arm dynein, and these have been suggested to regulate PKA activity (Inaba *et al*., 1993, 1998). Interestingly, a proteasome-containing structure extends to the plasma membrane, suggesting a linkage from the plasma membrane to the outer arm dynein (Inaba *et al*., 1998). *Chlamydomonas* axonemes contain casein kinase I associated with doublet microtubules (Yang and Sale, 2000). Although casein kinase I is present in mammalian sperm, it is not clear whether it is associated with sperm flagellar axonemes (Chaudhry *et al*., 1991).

Tyrosine phosphorylation of sperm proteins also plays crucial roles in the regulation of flagellar motility in sperm (Hayashi *et al*., 1987; Visconti *et al*., 1995; Dey and Brokaw, 1991; Si and Okuno, 1999). Several proteins have been reported to be phosphorylated, possibly in relation to motility activation of sperm, including hexokinase (Carrera *et al*., 1996), AKAP82 (Carrera *et al*., 1996), a 15-kDa protein in the base of the flagellum (Jin *et al*., 1994), glycogen synthase kinase (Vijayaraghavan *et al*., 2000), and many uncharacterized proteins. Most tyrosine phosphorylation is regulated by cAMP-dependent protein kinase (Hayashi *et al*., 1987; Visconti *et al*., 1995). Although tyrosine phosphorylation of these proteins seems to be associated with sperm motility, no evidence has been reported for the presence of tyrosine-phosphorylated axonemal components.

Both type 1 and 2A protein phosphatases are associated with flagellar axonemes in *Chlamydomonas* (Yang *et al*., 2000). However, both were extracted from sperm fla-

gella in salmonid fish sperm using Triton X-100 (Inaba, 2002), although type I protein phosphatase appears to be associated with the axonemes and regulate sperm motility in fowl (Ashizawa *et al*., 1998). Ca^{2+}-dependent type 2B protein phosphatase was reported to be bound to the axoneme in sea urchin and mammalian sperm and may be involved in the regulation of dynein phosphorylation (Tash *et al*., 1988).

Radial spokes and central pairs contain AKAP as a component as described above. In addition to radial spokes, several AKAPs are known to be present in mammalian sperm, such as AKAP82, AKAP110, and AKAP220 (Vijayaraghavan *et al*., 1999; Reinton *et al*., 2000). AKAP82 is a major component of the fibrous sheath of mammalian sperm flagella (Carrera *et al*., 1994). AKAP28 has recently been identified as a component of airway ciliary axonemes in human (Kultgen *et al*., 2002). This protein is expressed at high levels in the testis, suggesting that it is also an axonemal component of sperm flagella, although its precise localization remains to be determined.

Changes in the direction of sperm movement are caused by modulation of the beating of flagellar waves, a process that involves Ca^{2+}-dependent changes in flagellar asymmetry (Brokaw, 1979). The RS/CP system is a target of Ca^{2+} (Bannai *et al*., 2000; Smith, 2002) through calcium-binding proteins, such as CaM, several CaM-binding proteins (Wasco *et al*., 1989; Ueno *et al*., 2003), or calsequestrin-like Ca^{2+}-binding protein (Berruti and Porzio, 1990). As described above, certain Ca^{2+}-binding proteins are components or associated proteins of outer arm dynein, which may also be involved in the asymmetrical axonemal movement.

Outer dense fiber and fibrous sheath in mammalian sperm: ODF has been reported to be composed of several cysteine- and proline-rich, intermediate filament-like proteins (Olson and Sammons, 1980; Vera *et al*., 1984; Oko, 1988). At least 14 polypeptides have been identified as ODF components and are believed to play roles in maintenance of this passive elastic structure (Oko, 1988). Some ODF components and associated proteins have been cloned. ODF1 contains leucine zipper motifs which appear to be responsible for self-interaction and interaction with other ODF components (Shao *et al*., 1997). FS is composed of at least 18 polypeptides (Oko, 1988) and seems to serve as a scaffold for several enzymes for energy metabolism and as a signaling molecule for sperm motility. FS is known to be comprised of several proteins, including AKAPs (AKAP3, AKAP4, TAKAP80), hexokinase (HK1-S), and Rho-binding protein rhophilin and its binding protein ropporin (reviewed in Eddy *et al*., 2003). Interestingly, rhophilin is located on the inner surface of FS facing the axoneme (Fujita *et al*., 2000). This may be related to the tyrosine phosphorylation of FS components, such as HK-1 and AKAP3 (Carrera *et al*., 1994; Mori *et al*., 1998) or to FS sliding (Si and Okuno, 1995).

II. Molecules involved in motility regulation

Sperm motility changes prior to fertilization (Fig. 3). Sperm are immotile in testis even after the completion of spermiogenesis. In animals that undergo external fertilization, sperm initiate motility upon spawning triggered by extracellular ionic changes or substances released from the egg. In fish, sperm appear to undergo maturation in the sperm duct, which is a prerequisite for motility initiation (Morisawa and Morisawa, 1988). Mammalian sperm mature to acquire the ability for motility while passing through the epididymis. Sperm cells ejaculated into the female reproductive tract undergo capacitation. Capacitated sperm show vigorous movements called hyperactivation (Yanagimachi, 1970). In almost all organisms, substances from the egg induce changes in the direction of sperm movement, resulting in chemotaxis toward the egg (Miller, 1985; Cosson, 1990).

Activation of sperm flagellar motility is triggered by reception of ionic changes or substances from the female reproductive tract or the egg. It involves activation of both energy metabolism and the motile apparatus. The former includes many enzymes for ATP production and ATP regeneration (Mohri, 1957; Mita and Yasumasu, 1983; Tombes

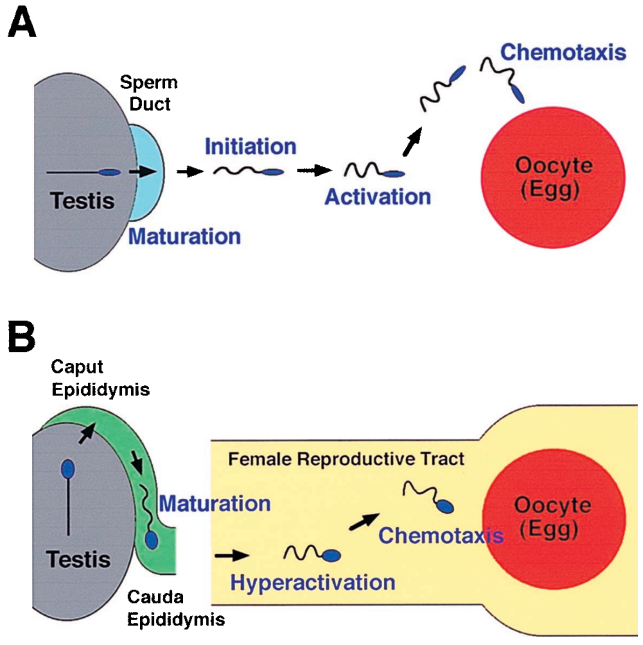

Fig. 3. Changes in sperm motility prior to fertilization. *A*, Sperm from animals with external fertilization initiate motility at spawning, triggered by extracellular ionic changes or by substances released from the egg. In fish sperm, specific changes or maturation of motility occur while passing through the sperm duct. Factors released from the egg cause sperm to undergo activation and chemotaxis toward the egg. *B*, Sperm from animals with internal fertilization show no motility just after spermiation into the caput epididymis. While passing through the epididymis, they undergo maturation and acquire motility. After ejaculation into the female reproductive tract, their motility changes dramatically. The sperm undergo capacitation and exhibit hyperactivation of motility. A specific substance in the tract shows chemotactic activity toward the sperm.

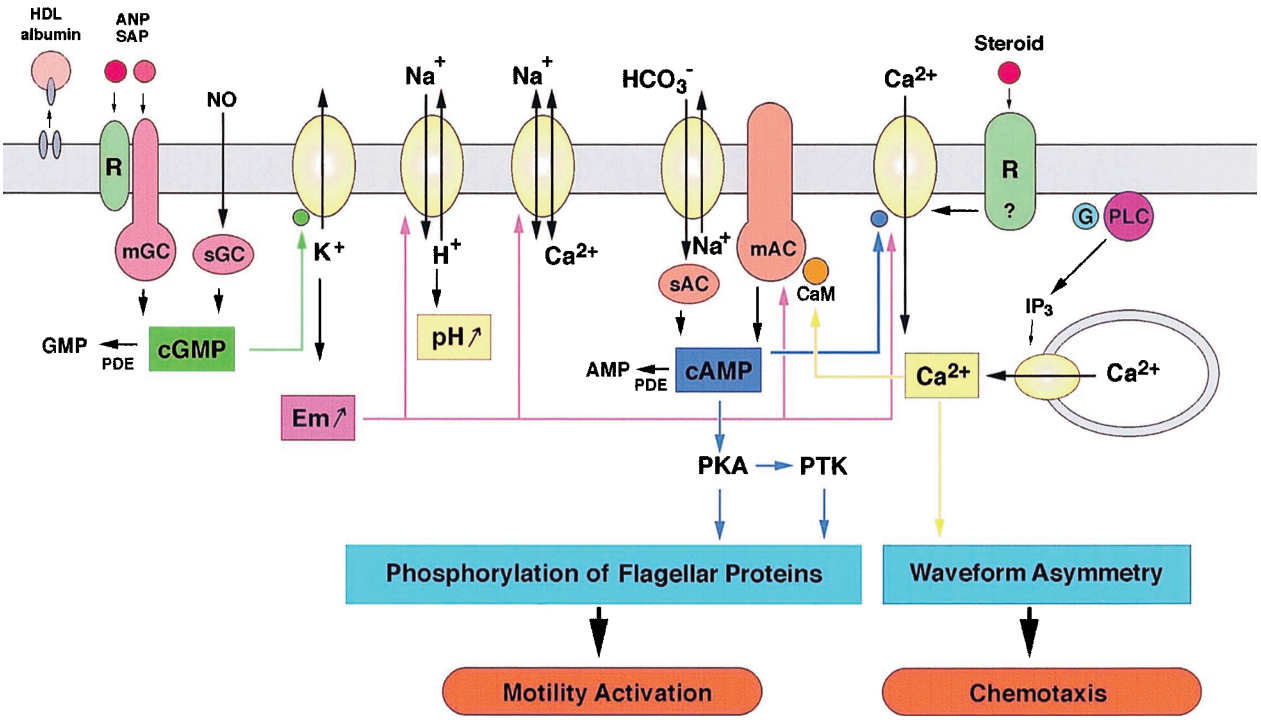

Fig. 4. Transmembrane signaling pathway for the activation of sperm motility. The molecules identified in sperm from multiple organisms were put together in the scheme. The cholesterol efflux shown at the left is suggested to participate in changes of membrane fluidity at capacitation in mammals. Sperm-activating peptide (SAP) binds directly or via a receptor to transmembrane guanylyl cyclase (mGC) and activates it. Nitric oxide (NO) activates a soluble guanylyl cyclase (sGC). cGMP activates a specific type of K^+ channel, and consequently the membrane potential is hyperpolarized. Membrane hyperpolarization stimulates several voltage-gated channels or ion exchangers. The Na^+/H^+ exchanger induces intracellular alkalization, which raises enzymatic activity of dynein. Ca^{2+} efflux through the Na^+/Ca^{2+} exchangers plays a role in keeping intracellular Ca^{2+} low in sea urchin sperm. In fish, the converse action of this channel causes Ca^{2+} influx. In *Ciona* and salmonid fish, the production of cAMP is closely coupled with K^+-dependent membrane hyperpolarization. In carp, hyperpolarization activates Ca^{2+}-channels to cause Ca^{2+} influx. Transmembrane adenylyl cyclase (mAC) regulated by CaM was identified in sea urchin sperm. Soluble adenylyl cyclase is activated by bicarbonate (HCO_3^-), which may be transported into the cell by Na^+/HCO_3^- cotransporter in mammals. The amounts of both cGMP and cAMP are negatively regulated by phosphodiesterases (PDEs). Some Ca^{2+}-channels in sperm are regulated by cAMP. Steroids, such as progesterone, induce Ca^{2+} influx possibly through its hypothetical receptor (R). Release of Ca^{2+} from store-operated Ca^{2+} channels is likely to be induced by IP_3 produced by phospholipase C (PLC), which is activated by binding G protein (G). Protein phosphorylation by cAMP-dependent protein kinase (PKA), as well as by protein tyrosine kinase (PTK), modulates flagellar protein to activate motility. Increases in intracellular Ca^{2+} cause the activation of other channels or signaling molecules, as well as modulation of flagellar wave asymmetry, which causes changes in the direction of movement and ultimately leads to chemotaxis of sperm toward the egg.

and Shapiro, 1985) and is not discussed in detail in this review. The latter involves a signaling pathway from the plasma membrane to the axoneme. Several kinds of ionic channels and receptors along with the enzymes for cyclic nucleotide synthesis have been identified as molecules involved in this signaling pathway (Fig. 4). The final target of this signaling pathway is the alternation of axonemal movement, including activation of dynein by protein phosphorylation and modulation of flagellar bend asymmetry.

Receptors for extracellular sperm-activating substances

The egg jelly-associated peptide in the sea urchins *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus*, called speract (or sperm activating peptide I; SAP-I), is a peptide consisting of ten amino acids. This molecule binds to a specific sperm surface receptor, resulting in increased sperm motility and respiration rate (Suzuki *et al*., 1981; Hansbrough and Garbers, 1981). Receptors for the peptide were identified on the sperm membrane in *H. purcherrimus* (Shimizu *et al*., 1994) and in *S. purpuratus* (Dangott and Garbers, 1984) with molecular masses of 71 kDa and 77 kDa, respectively. The binding of speract to the receptor activates a guanylyl cyclase on the plasma membrane (Bentley *et al*., 1988). A 14-amino acid peptide, resact (SAPIIA), from another species of sea urchin, *Arbacia punctulata*, binds directly to guanylyl cyclase on the sperm plasma membrane (Suzuki *et al*., 1984; Singh *et al*., 1988). Likewise, asterosap, a sperm-activating peptide from starfish, binds to a 130-kDa membrane protein that is likely to be a guanylyl cyclase (Nishigaki *et al*., 2000).

Two proteins that activate sperm motility have been identified in herring: one has a small molecular mass (~8 kDa) (Oda *et al*., 1998), while the other is a 105-kDa glycoprotein present on the micropyle of the egg (Vines *et al*., 2002). Receptors for these two proteins have not been identified. Interestingly, chordates might have developed the use

of steroids as molecules to trigger modulation of sperm motility. An oocyte maturation-inducing hormone, 17α, 20β-dihydroxy-4-pregnen-3-one, is responsible for the maturation of sperm motility in the sperm duct of salmonid fish (Miura *et al*., 1992). In the ascidians *Ciona intestinalis* and *C. savignyi*, a novel sulfate steroid, SAAF (sperm-activating and attracting factor), induces sperm activation and chemotaxis, but the receptor on sperm remains to be identified (Yoshida *et al*., 2002). Progesterone activates sperm motility in mammals by inducing Ca^{2+} influx (Uhler *et al*., 1992). On the other hand, sperm appear to receive signals in a manner similar to chemical reception of sensory organs. For example, specific olfactory receptors are expressed on the midpiece of sperm flagella (Vanderhaeghen *et al*., 1993). Recently, an odorant receptor, hOR17-4, was shown to be involved the chemotaxis of human sperm (Spehr *et al*., 2003).

Ion channels and cyclic nucleotide cyclases

Binding of egg-derived substances to the sperm receptor or several kinds of ionic changes around sperm induce membrane hyperpolarization, Ca^{2+} influx, increases in intracellular pH, and activation of several enzymes related to the subsequent signal cascade for activation of sperm motility (Fig. 4). The membrane proteins involved in this process include voltage-dependent or cGMP-gated K^+ channels, Na^+/H^+ and Na^+/Ca^{2+} exchangers, and Na^+/HCO_3^- cotransporters. There is accumulating evidence for the presence of several ion channels on sperm, but only a few of these channels have been characterized well in relation to flagellar motility at the molecular level. Following the reception of extracellular signals, cyclic nucleotide cyclases are activated to induce transient increases in cGMP and cAMP, both of which are essential for intracellular signal transduction to activate axonemal movement. Ca^{2+} influx from outside the sperm through Ca^{2+} channels or the Na^+/Ca^{2+} exchanger, or from intracellular stores through store-operated Ca^{2+}-channels (SOC) causes the activation of other channels or enzymes, as well as the changes in flagellar wave asymmetry necessary for chemotaxis toward the egg.

K^+ channels: A decrease in extracellular K^+ concentration triggers the initiation of sperm motility in salmonid fish. In sea- or freshwater fish, such as carp, a change in osmolality triggers the initiation of sperm motility (Morisawa, 1994). K^+-dependent membrane hyperpolarization is caused by spawning into different ionic conditions in both cases (Takai and Morisawa, 1995; Kho *et al*., 2001; Krasznai *et al*., 2000). Membrane hyperpolarization has also been observed in *Ciona* sperm in which K^+ permeability is increased by SAAF (Izumi *et al*., 1999). Membrane hyperpolarization induces an increase in intracellular Ca^{2+} concentration and the activation of adenylyl cyclase (Kho *et al*., 2001; Izumi *et al*., 1999), which induces a transient increase in intracellular cAMP concentration. The K^+ channel has not yet been isolated from teleost or Ciona sperm, but it may be similar to the channel reported in Paramecium (Shultz *et al*., 1992; Izumi *et al*., 1999).

Intracellular levels of cyclic nucleotides increase at activation of sperm motility in most animals. Following the activation of guanylyl cyclase (GC) by binding of SAP, cGMP is synthesized and causes activation of a cGMP-gated K^+ channel, resulting in K^+ efflux and membrane hyperpolarization in sea urchin sperm (Babcock *et al*., 1992; Galindo *et al*., 2000). A cation channel, SPIH, has been cloned in sea urchin and shown to belong to the hyperpolarization-activated and cyclic nucleotide-gated K^+ channel (HCN) family (Gauss *et al*., 1998). SPIH has six putative transmembrane helices, a pore region, and cyclic nucleotide binding sites. SPIH is localized along the flagellum as a ~97-kDa phosphorylated form and may play roles in the activation of sperm motility.

Guanylyl cyclase: Transmembrane guanylyl cyclase (mGC) is a homodimeric glycoprotein. Peptides released from the egg, *i.e.*, resact in sea urchin and asterosap in starfish, can bind to a guanylyl cyclase to induce cGMP synthesis. In *Arbacia* sperm, binding of speract to the receptor activates guanylyl cyclase, resulting in cGMP production. Some phosphatases or phosphodiesterases may be involved in the rapid inactivation of GC or cGMP, respectively (Garbers, 1989; Suzuki, 1995). Some forms of guanylyl cyclase recognize atrial natriuretic peptides (ANP) (Garbers, 1991). Specific binding of ANP was detected in human sperm (Silvestroni *et al*., 1992).

The soluble form of guanylyl cyclase (sGC) is a heterodimeric hemoprotein consisting of α and β subunits. sGC is mainly activated by nitric oxide (NO) present in the female reproductive tract in the mouse (Burnett *et al*., 1995). Sperm also express NO synthase (NOS) activity and synthesize NO by utilizing several NO donors (Rosselli *et al*., 1996; Revelli *et al*., 2002).

Adenylyl cyclase: Intracellular cAMP is synthesized by adenylyl cyclase (AC). Cell membrane-bound AC with transmembrane domains (mAC) is regulated by G protein or Ca^{2+}/CaM and has been characterized extensively in somatic cells. In sea urchins, a 190-kDa CaM-associated adenylyl cyclase was identified and shown to be localized mainly on the proximal half of the flagellum (Bookbinder *et al*., 1990). Some types of Gα subunit are apparently localized in the flagella of mammalian sperm and may be involved in the modulation of adenylyl cyclase activity (Baxendale and Fraser, 2003). In human sperm, a CaM-dependent phosphodiesterase (PDE) is localized along the flagellum and seems to be involved in the regulation of flagellar cAMP level (Lefievre *et al*., 2002).

Bicarbonate (HCO_3^-) is involved in maturation of sperm motility in salmonid fish and mammals (Morisawa and Morisawa, 1988; Okamura *et al*., 1985), although it has been shown to inhibit sperm motility in flatfish through the action of carbonic anhydrase (Inaba *et al*., 2003). Recently,

a unique soluble adenylyl cyclase (sAC) was cloned and shown to be activated in a bicarbonate-dependent, G protein-independent manner (Buck *et al*., 1999). As sperm AC is activated by extracellular bicarbonate ions (Okamura *et al*., 1985), production of cAMP in sperm is likely to be carried out, at least in part, by soluble AC. A Na^+/HCO_3^- cotransporter is present in mouse sperm and could participate in the transport of HCO_3^- into sperm (Demarco *et al*., 2003). Sperm hyperactivation appears to be modulated by reactive oxygen species (ROS) (Aitken and Fisher, 1994; de Lamirande *et al*., 1997). The target of ROS is unclear, but has suggested to be a factor involved in cAMP production, most likely adenylyl cyclase (de Lamirande and Gagnon, 1999).

Na^+/H^+ and Na^+/Ca^{2+} exchangers: Influx of Na^+ is essential for the motility of sea urchin sperm. The spawning of sperm into seawater involves intracellular alkalization through the Na^+/H^+ antiport that may induce activation of motility (Nishioka and Cross, 1978; Christen *et al*., 1983). A voltage-sensitive Na^+/H^+ exchanger has been suggested to be activated by K^+-dependent membrane hyperpolarization. Efflux of H^+ induces intracellular alkalization, which activates dyneins (Christen *et al*., 1983; Johnson *et al*., 1983; Lee, 1985). On the other hand, a 90-kDa K^+-dependent Na^+/Ca^{2+} exchanger, suNCKX, was identified and cloned in sea urchin (Su and Vacquier, 2002). This molecule is localized on the plasma membrane of sperm flagella and may maintain a low level of intracellular Ca^{2+}. It possesses two PKA sites and a His-rich region in the cytoplasmic loop, suggesting regulation of the channel by cAMP-dependent phosphorylation and metal binding. When herring sperm initiate motility upon reception of the 105-kDa glycoprotein, SMIF, membrane hyperpolarization occurs and subsequently both voltage-dependent Ca^{2+} channels and Na^+/Ca^{2+} exchangers appear to be activated for Ca^{2+} influx (Vines *et al*., 2002). In this case, the activity of the Na^+/Ca^{2+} exchanger should operate in reverse. A putative 120-kDa Na^+/Ca^{2+} exchanger is localized along the flagella of herring sperm (Vines *et al*., 2002).

Ca^{2+} channels: Influx of external Ca^{2+} is known to be essential for activation of sperm motility. Several types of Ca^{2+} channels are involved in this process (Darszon *et al*., 2001). A cAMP-regulated Ca^{2+} channel was identified and suggested to participate in Ca^{2+}-dependent depolarization in sea urchin sperm (Cook and Babcock, 1993). A putative 686-amino acid sperm cation channel, CatSper, has been isolated and cloned. CatSper is located in the principal piece of the flagella in mouse sperm and is involved in the regulation of sperm motility by mediating cAMP-induced Ca^{2+} influx (Ren *et al*., 2001). In carp sperm, membrane hyperpolarization removes inactivation of Ca^{2+} channels and causes Ca^{2+} influx at initiation of sperm motility. However, the process of the activation of motility seems to be independent of cAMP (Krasznai *et al*., 2000). On the other hand, homologs of *Drosophila* transient receptor potential channels (Trp) are present in mouse sperm. Among them, Trp1 and Trp3 are localized on sperm flagella, suggesting that Trps are involved in the Ca^{2+} influx required for activation of sperm motility (Trevino *et al*., 2001).

Ca^{2+} release from intracellular stores is involved in the modulation of sperm motility. Store-operated Ca^{2+} channels have been shown to be involved in hyperactivation of bull sperm (Ho and Suarez, 2001) and chemotaxis in *Ciona* sperm (Yoshida *et al*., 2003). Receptors for inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from intracellular stores, are localized on the flagella of mammalian sperm (Ho and Suarez, 2001a), suggesting that the Ca^{2+}-release signal for hyperactivation of sperm is IP_3.

Lipid raft as a potential signaling scaffold

There is accumulating evidence that compartmentalization of the sperm plasma membrane is closely associated with the regulation of sperm motility. Albumin-induced sperm capacitation accompanies the decrease in sterol level of the plasma membrane. The similarity of the effect of albumin to those of high-density lipoprotein (HDL) and β- cyclodextrins suggests that serum albumin affects sperm capacitation through cholesterol efflux (Travis and Kopf, 2002). In fact, a loss of cholesterol was observed from the sperm plasma membrane and appears to be associated with cAMP-dependent phosphorylation and with tyrosine phosphorylation of sperm proteins (Visconti *et al*., 1999).

The membrane subdomain rich in cholesterol and sphingolipids is called the “lipid raft.” Efflux of cholesterol during sperm capacitation may be related to the dynamics of the membrane lipid raft by increasing membrane fluidity (Trevis and Kopf, 2002), and ultimately the dynamics of signal molecules in the raft, such as receptors and ion channels. For example, cholesterol efflux induces an increase in protein tyrosine phosphorylation in mammalian sperm (Visconti *et al*., 1999). In addition, Trp1 is co-localized with caveolin-1, which is a major component of caveolae, a subset of the lipid raft (Trevino *et al*., 2001). Bicarbonate, cAMP, and Ca^{2+} were reported to affect cholesterol depletion and lipid architecture, suggesting the rearrangement of membrane proteins upon activation of motility (Harrison *et al*., 1996; Flesch *et al*., 2001).

III. Genome-wide and proteomic approaches to comprehensive understanding of flagellar architecture

The system of flagellar motility is composed of a sophisticated protein network. As described above, only a subpopulation of sperm flagellar proteins have been characterized at the molecular level. The relationship between each protein and the spatial organization of each component have not been elucidated. The flagellum contains more than 400 proteins, less than half of which have been characterized. To understand the architecture and function of flagellar motility, extensive analyses of flagellar proteins are required.

Recent genome-wide and proteomic studies have made

it possible to extensively describe proteins expressed in a given type of cell. For example, immunoscreening of cDNAs by antibodies against axonemal proteins revealed 76 axonemal proteins, including novel proteins, in *Ciona intestinalis* (Padma *et al*., 2003). Mass spectrometric analysis in conjunction with two-dimensional gel electrophoresis identified a number of proteins in human airway cilia (Ostrowski *et al*., 2002). Analysis of tyrosine phosphorylated proteins in human sperm by tandem mass spectrometry identified proteins that are phosphorylated during capacitation (Ficarro *et al*., 2003). Application of these proteomic methods to analyses of flagellar compartments, such as the axoneme, plasma membrane, or lipid raft, may improve our overall understanding of how sperm cells are constructed and how the signaling network is involved in the rapid response of flagellar motility to changes in the ionic environment or to substances released from the egg.

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