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Calmodulin localization in mammalian spermatozoa

(immunofluorescence/acrosome reaction/television/sperm)

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ABSTRACT **The location of calmodulin in rabbit and guinea pig spermatozoa was determined by indirect immunofluorescence techniques. Spermatozoa that had not undergone the acrosome reaction exhibited four distinct regions of calmodulin-specific immunofluorescence: around the acrosome, in a band across the lower third of the head, and in two localized areas at the base and tip of the flagellum. In contrast, after the acrosome reaction, although other features of calmodulin distribution remained the same, the fluorescence associated with the anterior half of the head was notably absent. Instead, fluorescence was associated with the membranes that had separated from the sperm head. These findings suggest a potential role for calmodulin in the Ca^{2+}-dependent control of sperm activation, in sperm–egg fusion, and in microtubule disassembly processes in the flagellum.**

After ejaculation and introduction into the female reproductive tract, the mammalian spermatozoon undergoes a series of physiological and morphological changes that are both required for and facilitate the process of fertilization. The first of these processes, capacitation, results in a loss of cell coating material and an increased permeability of the plasma membrane to metal ions (1–3). This influx of ions triggers a second process, termed the "acrosome reaction," that is characterized by extensive vesiculation of the plasma and outer acrosomal membranes (1). This vesiculation results in the eventual loss of these membranes and the release of the hydrolytic contents of the acrosome which aid in the penetration of the ovum vestments. The acrosome reaction, essential for fertilization, is dependent upon calcium influx after capacitation (3, 4). Extracellular calcium is required for the vesiculation process (2), and agents that promote the rate of calcium influx also promote the rate of initiation of the acrosome reaction (3). Although the case for calcium involvement in acrosomal activation is well documented, little is known of the biochemical mechanisms that link calcium to the subsequent cellular events.

The regulatory protein calmodulin, ubiquitous in eukaryotic systems, activates, in a calcium-dependent manner, various enzymatic activities (5–16) and appears to participate in such cellular processes as mitosis (17) and synaptosomal neurotransmitter secretion (18, 19). In light of its ability to regulate cellular processes, we thought it reasonable that calmodulin might serve as the link between calcium influx and the acrosome reaction. To test this hypothesis, we initially surveyed the calmodulin content of spermatozoa isolated from a wide variety of species and determined that calmodulin was indeed present in surprisingly high levels in all species of spermatozoa examined (20). Utilizing conventional techniques for sperm fractionation, we discovered that all of the calmodulin detectable by biological assay in rabbit spermatozoa was localized within the cytosolic or acrosomal portions of the head (20), an observation consistent with its potential role in acrosomal activation. In order to supplement our initial findings and to gain further insight into the question of calmodulin-dependent regulation of sperm activation, we attempted to localize calmodulin in rabbit and guinea pig spermatozoa, with and without acrosomes, by using immunofluorescence techniques. The results of these immunofluorescence studies are reported here.

MATERIALS AND METHODS

Sources of Proteins and Antibodies. IgG directed against rat testes calmodulin was prepared from goats by the method of Dedman *et al.* (22) and was the generous gift of A. R. Means and J. R. Dedman. Porcine brain calmodulin was purified by the method of Charbonneau and Cormier (21). Fluorescein-conjugated rabbit anti-goat IgG was purchased from Cappel Laboratories, Cochranville, PA.

Spermatozoa Collection. Guinea pig spermatozoa were routinely obtained by perfusion of extracted epididymides with Dulbecco's phosphate-buffered saline (P_i/NaCl). On occasion, to obtain rapid fixation of the spermatozoa, epididymides were flushed with 3.7% formaldehyde in P_i/NaCl.

Rabbit spermatozoa were collected, with the aid of an artificial vagina, into a vial containing 1.5 ml of a calcium-free buffer (140 mM NaCl/4 mM KCl/25 mM Hepes/7 mM $MgCl_2$/5 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH 6.3), which decreased the probability of initiating the acrosome reaction. When ejaculates were collected into P_i/NaCl, within minutes after collection, approximately 30% of the cell population was observed to have undergone an acrosome reaction. These were the cells examined for calmodulin localization in "acrosome-reacted" spermatozoa. For fixation, spermatozoa were treated with 3.7% formaldehyde in P_i/NaCl directly on slides or, alternatively, suspensions were centrifuged at 600 × *g* for 5 min, the seminal plasma was decanted, and the spermatozoa-containing pellet was resuspended in the fixative.

Immunofluorescence Labeling. All spermatozoa, suspended in saline, calcium-free buffer, or formaldehyde solution, were diluted to an appropriate concentration with P_i/NaCl and a drop of the resultant solution was applied to slides pretreated with 2% polylysine. The drop of spermatozoa suspension was then covered with a glass coverslip, and gentle pressure was applied to facilitate adhesion of the spermatozoa to the polylysine. Spermatozoa that had not previously been fixed with formaldehyde were then treated with 3.7% formaldehyde in P_i/NaCl for 10 min. All preparations were then rinsed in P_i/NaCl, treated with cold acetone (−80°C) for 3 min, and rinsed

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Abbreviation: P_i/NaCl, Dulbecco's phosphate-buffered saline.

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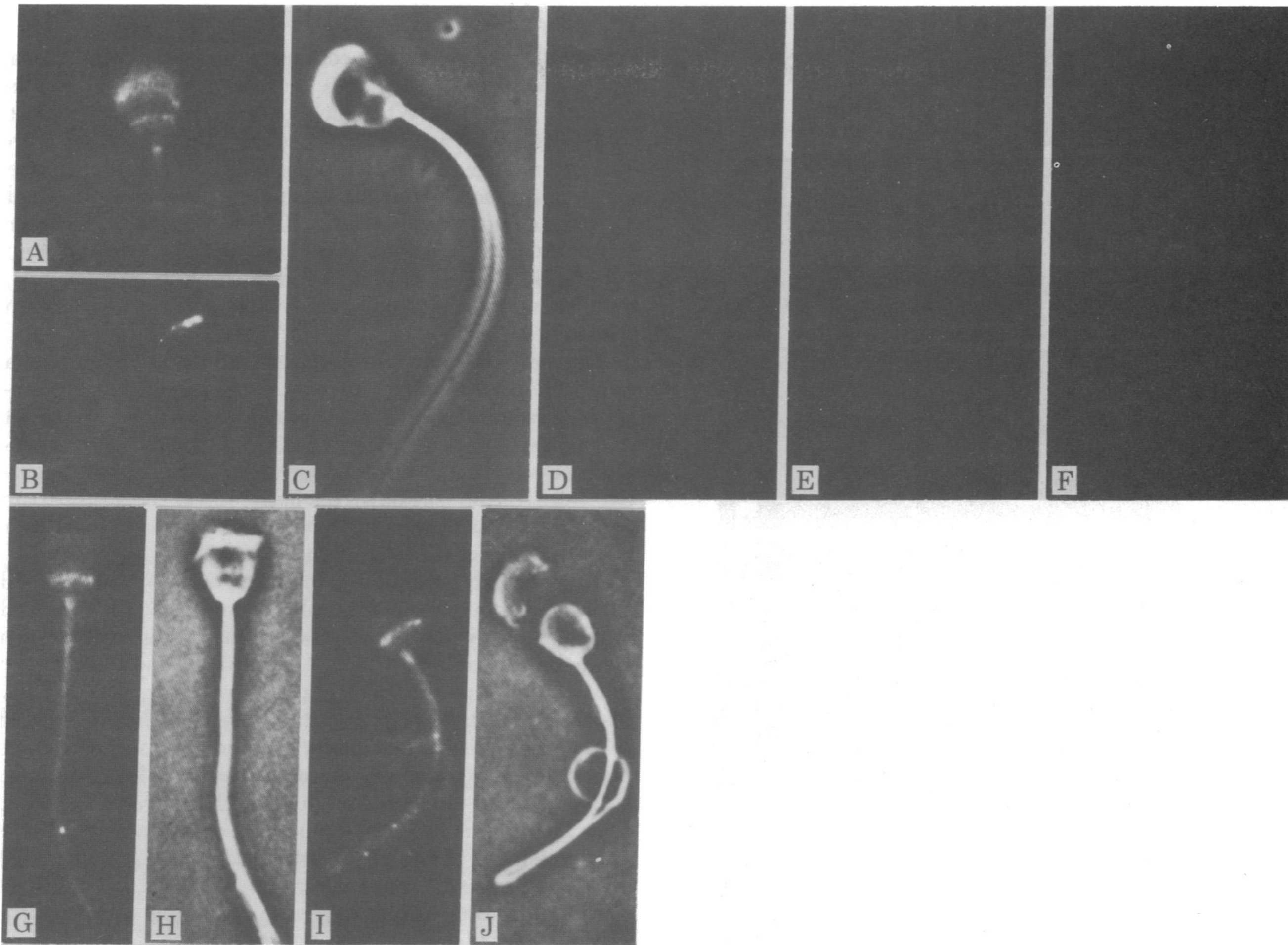

FIG. 1. Guinea pig spermatozoa. (Photographs in *D*, *E*, and *F* were made at the same exposure settings and development time as those in *A* and *B*; ×1300.) (*A*) Portion of an intact sperm treated with anticalmodulin antibody followed by fluorescein-labeled second antibody. Note fluorescence in the head at the acrosome, in a band further down the head, and in a bright spot at the junction with the tail. Another sperm can barely be discerned out of the plane of focus. (*B*) Portion of the tail of a sperm treated as in *A*. Fluorescence is evident at the tip. (*C*) Anoptral phase-contrast image of a control, intact sperm treated with nonimmune goat IgG instead of specific first antibody. (*D*) Fluorescence image of the same control cell shown in *C*. (*E*) Fluorescence image of a sperm treated with second antibody alone. (*F*) Fluorescence image of a sperm treated with anticalmodulin antibody preadsorbed with an excess of calmodulin and then exposed to fluorescein-tagged second antibody. (*G* and *H*) Fluorescence (*G*) and phase-contrast (*H*) images of a sperm with a partially lifted acrosome. Note that the lifted membranes are still somewhat fluorescent. (*I* and *J*) An acrosome-reacted sperm with a presumed acrosomal "cap" close by, viewed with fluorescence (*I*) and phase-contrast (*J*). The cap is only weakly fluorescent. In *G* and *I*, the band and the basal spot are still brightly fluorescent. The tip of the tail is either out of the picture (*G*, *H*) or is tangled in the rest of the tail (*I*, *J*).

again. To prevent subsequent nonspecific binding of immunoglobulins, formaldehyde- and acetone-treated preparations were incubated at room temperature for 30 min with whole rabbit serum, and all subsequent incubations were conducted in the presence of 1% rabbit serum.

After treatment with whole rabbit serum, a small drop (20 μl) of the calmodulin-specific antibody (50 μg/ml) was applied to the spermatozoa preparation. The drop was then covered with a glass coverslip and the treated preparation was allowed to incubate for 90 min at room temperature. The coverslip was removed and the preparation was rinsed in P_i/NaCl containing 1% rabbit serum. Preparations were then flooded with an excess of fluorescein-conjugated rabbit anti-goat IgG diluted 1:300 in P_i/NaCl/1% rabbit serum and incubated in the dark at room temperature for 90 min. After incubation with the second antibody the preparations were again exhaustively washed in P_i/NaCl/1% rabbit serum. A drop of 90% (vol/vol) glycerol in P_i/NaCl was then applied to the slide and the sample was covered with a glass coverslip. Controls for the specificity of immunofluorescence included (*i*) treatment of fixed spermatozoa with fluorescein-labeled second antibody alone, (*ii*) replacement of calmodulin-specific goat IgG with nonimmune goat IgG (100 μg/ml), and (*iii*) pretreatment of calmodulin-specific antibody with an excess of porcine brain calmodulin (1 mg/ml).

Microscopy. Cells were examined with a Reichert Zetopan microscope equipped with anoptral phase-contrast and epifluorescence illumination. An FITC/BG38 exciter filter combination, together with a 490-nm barrier filter/beam splitter were used for fluorescence observation. Fluorescence and phase-contrast images were visualized with the aid of a double intensified vidicon television camera (Venus Scientific, Farmingdale, NY) and a high-resolution monitor (Panasonic, Atlanta, GA) as described by O'Kane and Palevitz (23). Exposures were made on Kodak SO-115 35-mm film either directly from the monitor or during playback of images stored on a 3/4-inch video time lapse recorder run at real time (Nippon Electric Co., Elk Grove, IL). The film was exposed for 1/2 to 1/8 sec and processed in Kodak HC110(D) developer with Factor 8 (Min Max, Harbor City, CA) added.

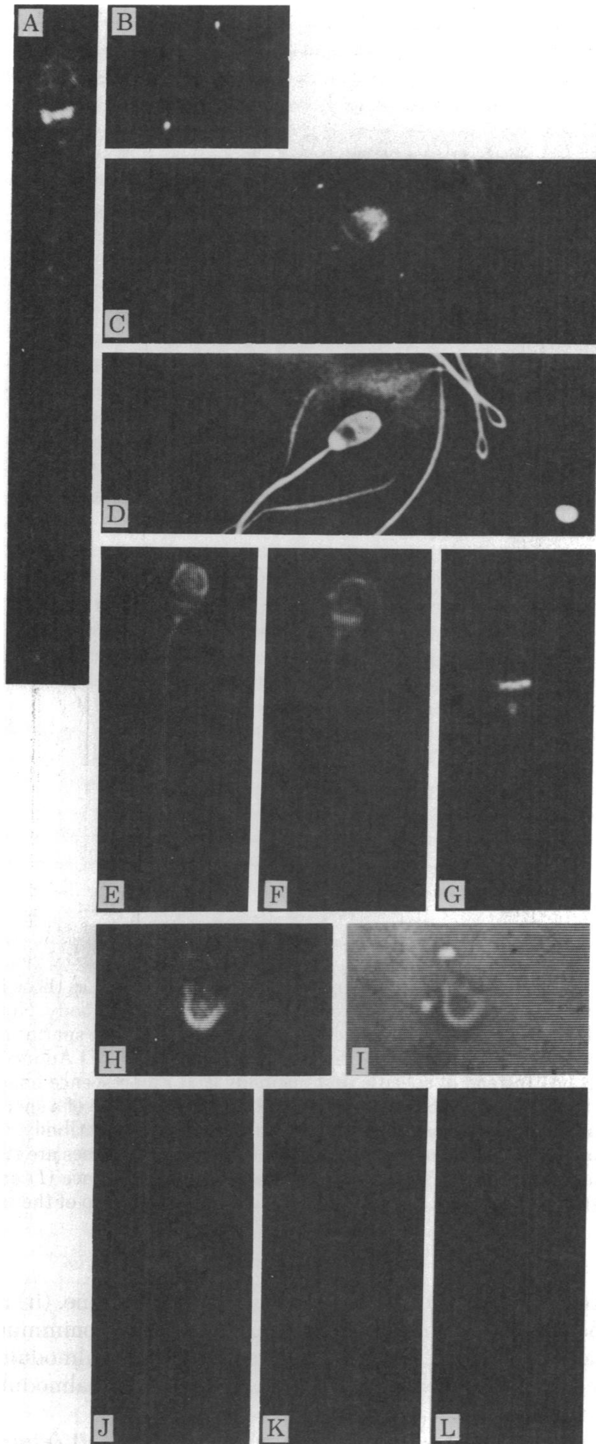

FIG. 2. Rabbit spermatozoa. The sperm in A–I were treated with anticalmodulin antibody as described in the text and in Fig. 1*A*. (*A*) Fluorescence image of a complete, intact sperm, showing bright fluorescence in three regions around the head and at the tip of the tail. (×1700.) (*B*) Portions of two sperm tails. Note fluorescence at the tips. (×1700.) (*C* and *D*) Fluorescence (*C*) and phase-contrast (*D*) images of an intact sperm head and portions of a few tails. Note fluorescence at the tip of the tails and in three regions of the head. (×1080.) (*E–G*) Fluorescence images of sperm in various stages of the acrosome reaction. In *G*, the acrosome is completely gone. (*E* and *F* ×1150; *G*, ×1700.) (*H* and *I*) Fluorescence (*H*) and phase-contrast (*I*) images of a free acrosomal cap. (×1700.) (*J–L*) Fluorescence images of intact, control sperm made with the photographic procedure outlined in Fig. 1*D*. (×1080.) (*J*) Sperm cell treated with nonimmune goat IgG instead of specific first antibody; (*K*) sperm cell treated with fluorescein-tagged second antibody alone. (*L*) Sperm cell treated with anticalmodulin antibody preadsorbed with an excess of calmodulin and then with second antibody.

RESULTS

Fig. 1*C* is an anoptral phase-contrast image of a typical guinea pig spermatozoon. The large, refractile, acrosomal cap is especially prominent. When such sperm were treated with goat antibody directed against rat testes calmodulin and then with fluorescein-tagged second antibody, four principal areas of the sperm appeared fluorescent: the anterior region of the head corresponding to the acrosome, a band-like region in the lower half of the head, and spots at the base and tip of the tail (Fig. 1 *A* and *B*). The rest of the tail and the midpiece also stained, but this fluorescence usually was weaker and more variable in occurrence.

When rabbit sperm were processed in the same manner, a nearly identical staining pattern was observed, taking into account the somewhat different overall morphology of the rabbit sperm head compared to that of the guinea pig (Fig. 2). Fluorescence was found over the acrosome, in a band approximately two-thirds of the way down the head, and as spots at the base and tip of the tail, with weak fluorescence occasionally seen over the remainder of the tail and midpiece (Fig. 2 *A–C*).

Although only part of the acrosome of both species appeared to be stained in any one focal plane, careful focusing showed that fluorescence was associated with the entire acrosome down to the bright band seen in Figs. 1*A* and 2 *A* and *C*. We have now found this pattern in both species over the course of many experiments. In some preparations, free heads and isolated intact tails have also been observed, and the staining of these was identical to the corresponding pattern in whole cells (e.g., a bright spot at either end of an isolated tail.)

In sperm that had undergone the acrosome reaction, the fluorescence in the anterior part of the head was missing but remained in the other three areas (Figs. 1 *I* and *J* and 2*G*). In sperm exhibiting various degrees of acrosome shedding, the fluorescent material in the anterior region of the head corresponded to the partially lifted membranes seen in phase contrast (Figs. 1 *G* and *H* and 2 *E* and *F*). Often, apparent free acrosome caps could be found, and these too were fluorescent (Fig. 2 *H* and *I*). However, the fluorescence of partially lifted membranes, as well as free caps, often was weaker than that seen in the intact sperm (Figs. 1 *G* and *I* and 2*F*).

In contrast to the distinct fluorescence described above, control sperm that had been treated with either nonimmune goat IgG (Figs. 1 *C* and *D* and 2*J*) or with second antibody alone (Figs. 1*E* and 2*K*) exhibited no staining. Sperm treated with specific antibody preadsorbed with an excess of porcine brain calmodulin exhibited greatly decreased or no fluorescence at all (Figs. 1*F* and 2*L*).

DISCUSSION

Our results indicate that calmodulin is localized in four principal regions of the mammalian spermatozoon. Our conclusion is reinforced by the observations that the same four regions are stained with antibody in the sperm of two different species and that the localization appears to be specific based on the lack of staining in three different control experiments.

The relative preponderance of calmodulin in the anterior portion of the sperm head is consistent with our earlier findings (20), but the localization in the tail was not suspected in that study. Although from these studies it is impossible to identify which subcellular components or structures contain calmodulin and which do not, and definitive statements must await results of localization at the electron microscope level, certain con-

clusions can be drawn now. Because the events of the acrosome reaction do not affect the sperm nucleus, and because fluorescence is lost upon activation, it is apparent that the fluorescence observed in the anterior part of the head of non-acrosome-reacted spermatozoa is not nuclear in origin. However, several other explanations are possible.

In non-acrosome-reacted spermatozoa, calmodulin may be localized on either the plasma or the outer acrosomal membrane, in the cytoplasm between the two membranes, or in the acrosome itself. During the course of the acrosome reaction, vesiculation occurs between the plasma and outer acrosomal membranes. In time, most of the two membranes separate from the remainder of the sperm. The remnants of these membranes can often be observed as a cap lifted away or disassociated from the sperm head. These caps (Fig. 2 *H* and *I*) are fluorescent, indicating the presence of bound calmodulin. It is possible that either calmodulin is associated with the plasma or outer acrosomal membrane in non-acrosome-reacted spermatozoa or becomes associated with one of these membranes in response to the influx of calcium that occurs after capacitation. Although these "caps" are fluorescent, the intensity of staining is greatly reduced compared to that of sperm with intact acrosomes. This suggests that a significant portion of calmodulin is also released from the spermatozoa during the course of activation.

It is possible that calmodulin is present in the acrosome itself as well as associated with the acrosomal membrane. Release of acrosomal calmodulin might then account for the loss of fluorescence seen in heads after activation. However, such a localization does not readily account for why residual fluorescence is present in lifted caps but none is observed in the newly formed plasma membrane (formerly the "inner" acrosomal membrane) unless we suppose that acrosomal calmodulin is preferentially associated with the "outer" acrosomal membrane.

Although calmodulin may be located in the acrosome, our data also are consistent with the hypothesis that in non-acrosome-reacted spermatozoa the inactive or calcium-free form of calmodulin is localized in the cytoplasm, between the outer acrosomal and plasma membranes. In response to the calcium influx associated with the acrosome reaction, calcium might bind to calmodulin and a portion of the Ca–calmodulin complex could then associate with components of either the plasma or outer acrosomal membrane. The latter process could trigger the acrosome reaction. In time, plasma and outer acrosomal membranes containing bound calmodulin would become detached from the spermatozoa and fluorescent caps would be observed. Furthermore, because the contents of the cytoplasm are released into the medium during the acrosome reaction, a significant amount of unbound calmodulin would become lost to immunofluorescent detection. This is indeed what we observed. These processes, although still unproven, would not be unexpected in light of the redistribution of calmodulin known to occur in other cell types in response to changes in levels of intracellular calcium (24), in response to hormones (25), and during cellular processes such as mitosis (17). Because it is well established that calcium triggers the acrosome reaction (2, 3), and both our earlier studies (20) and this investigation confirm the presence of high levels of calmodulin in the area of the cell involved in the acrosome reaction, it seems likely that calmodulin serves as the link between calcium influx and the calcium-dependent acrosome reaction.

A second potentially important role for calmodulin is suggested by its localization in the posterior portion of the sperm. We have previously described the events of the acrosome reaction and noted that this process involves extensive vesiculation between the plasma and outer acrosomal membranes which results in the eventual shedding of those membranes. However, in mammalian spermatozoa, a small segment of those membranes that covers the posterior region of the acrosome does not undergo vesiculation and remains intact. This region is referred to as the equatorial segment (26). In electron micrographs (27), the plasma membrane at the posterior margin of this segment appears serrated, and this region might correspond to the bright band-like fluorescence we observe in the posterior half of both rabbit and guinea pig sperm. The events of sperm–egg fusion have led to the proposal that the equatorial segment plays an important role in sperm entry into the egg (28, 29). The entry of the sperm head into the egg requires the fusion of the plasma membranes of the egg and sperm. It is thought that this fusion process originates at or very near the equatorial segment (28, 29). The observations that Ca^{2+} is required for sperm–egg fusion (30) and that high levels of calmodulin are associated with the postacrosomal portion of the sperm suggest a potential role for calmodulin in the events of cell fusion during fertilization.

The significance of the intense fluorescence at the base and tip of the sperm tail is not immediately evident. It may, however, signify a role for calmodulin in the generation or breakdown of axonemal microtubules. It has recently been reported that calmodulin regulates the calcium-induced depolymerization of microtubules *in vitro* (17). Moreover, calmodulin has been specifically localized around neural microtubules (36) and in the mitotic spindle of dividing cells by immunocytochemical techniques, and intense staining was especially evident around the centrioles (17, 31). Thus, the fluorescence we observe at the base of the tail could be due to the presence of calmodulin around the basal body(ies) or associated material at that location. The localization at the tip could be related to the fact that central pair microtubules seem to insert in specialized structures associated with plasma membrane at the tips of cilia and flagella (32). Although the role of calmodulin at the tip of the tail is just as uncertain as that at the base, a function for both in axonemal assembly/disassembly (e.g., during sperm differentiation or disassembly after fertilization) must be considered. We note that microtubule assembly (including that of axonemal microtubules) occurs in a polar fashion with the distal end preferred severalfold *in vitro* (33–35). However, the central pair microtubules may actually grow at their proximal ends *in vivo* because of their insertion in the plasma membrane at the flagellar tip. Thus, microtubule assembly and disassembly in the axoneme may be bidirectional (32), which could account for calmodulin being located at the base and tip of the sperm tail. It must be stressed, however, that ideas on the role of calmodulin in sperm tails must remain speculative pending improved understanding of sites of microtubule flux in axonemes and of the role of calmodulin in flagellum formation or motility.

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