Primary Cilia in Locust Spermatocytes: Formation, Fate, and Possible Function

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During locust spermatocyte development, rudimentary cilia originate from all centrioles of the doubled prophasic centrosome, located in the centrosphere and specifically linked to the nuclear envelope. Membranous vesicles at the bases of the centrioles fuse to form an intracellular ampulla that contributes the ciliary membranes. Later in prophase the ampulla's membrane is integrated into the plasma membrane so that all primary cilia project into the extracellular medium and lose contact with the nucleus. As the centrioles remain ciliated on their way to their polar positions, we propose a mechanism for this migration which is based on this intimate association between centrosomes and plasma membrane, on membrane fluidity and on a contractile cell cortex. We noted a translocation of the ciliated centrioles below the cell surface at the metaphase/-anaphase transition which may be regarded as "deciliation" by light microscopy. Some possible explanations for the primary cilium's role in cell cycle regulation are suggested.

Introduction

Interest in the regulation of cell cycle events has increased considerably in the last decade [1–3]. As for mitosis, most attention has been directed to the regulation of anaphase onset [*e.g.* 4–6], possibly because the elucidation of the anaphase trigger could also hint at the mechanism of anaphasic chromosomal movements. Some recent investigations are suggestive of a special function of so-called primary cilia [7] in the regulation of anaphase [8–10].

A primary cilium is an immotile rudimentary cilium, temporarily generated mostly by one centriole of a diplosome in many cell types. It differs morphologically from the secondary cilia of specialized motile cells by the lack of the central tubules and the dynein arms [reviewed by 11].

In the course of our studies on anaphasic chromosomal movement in grasshopper spermatocytes [12, 13] we have chosen the osmium-ferrocyanide(OsFeCN)-staining technique [14] for EM-investigations. Due to its good preservation of fine structural details [15, 16], especially membranes [14], this fixation facilitates the tracing of primary cilia in spermatocytes I of *Locusta migratoria*. In view of their possible role in the regulation of any meiotic process, we attempted to study their formation and fate during spermatocyte development.

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Materials and Methods

Testes of fourth instar larvae of *Locusta migratoria* were cut into pieces in 0.45 ml Ringer's solution [17] and fixed by addition of 0.05 ml 25% glutardialdehyde. Further treatments were performed according to the OsFeCN-procedure of Hepler [14]. Ultrathin sections, cut with an LKB ultrotome II, were stained with lead citrate and examined with a Philips EM 300G operated at 80 kV. Since each piece of the testes includes several cut cysts, many spermatocytes of nearly the same developmental stage may be compared. Thus it becomes possible to trace the development of the organelle in question.

Results

Formation of primary cilia

In spermatocytes I of *Locusta migratoria* the centriole cycle starts with a quadruple formation, composed of the two parent centrioles and their daughter centrioles (Fig. 1a), which later divides into two diplosomes. At their bases, the centrioles associate with spherical vesicles (Fig. 1b), which fuse to form an intracellular ampulla. Bound to this membrane, the centrioles become basal bodies and organize microtubule doublets, so that four rudimentary cilia grow into the ampulla, using it as the source of ciliary membranes. Thus, already in early meiotic prophase, the centrosphere in the vicinity of the nucleus contains two closely situated diplosomes with all cen-

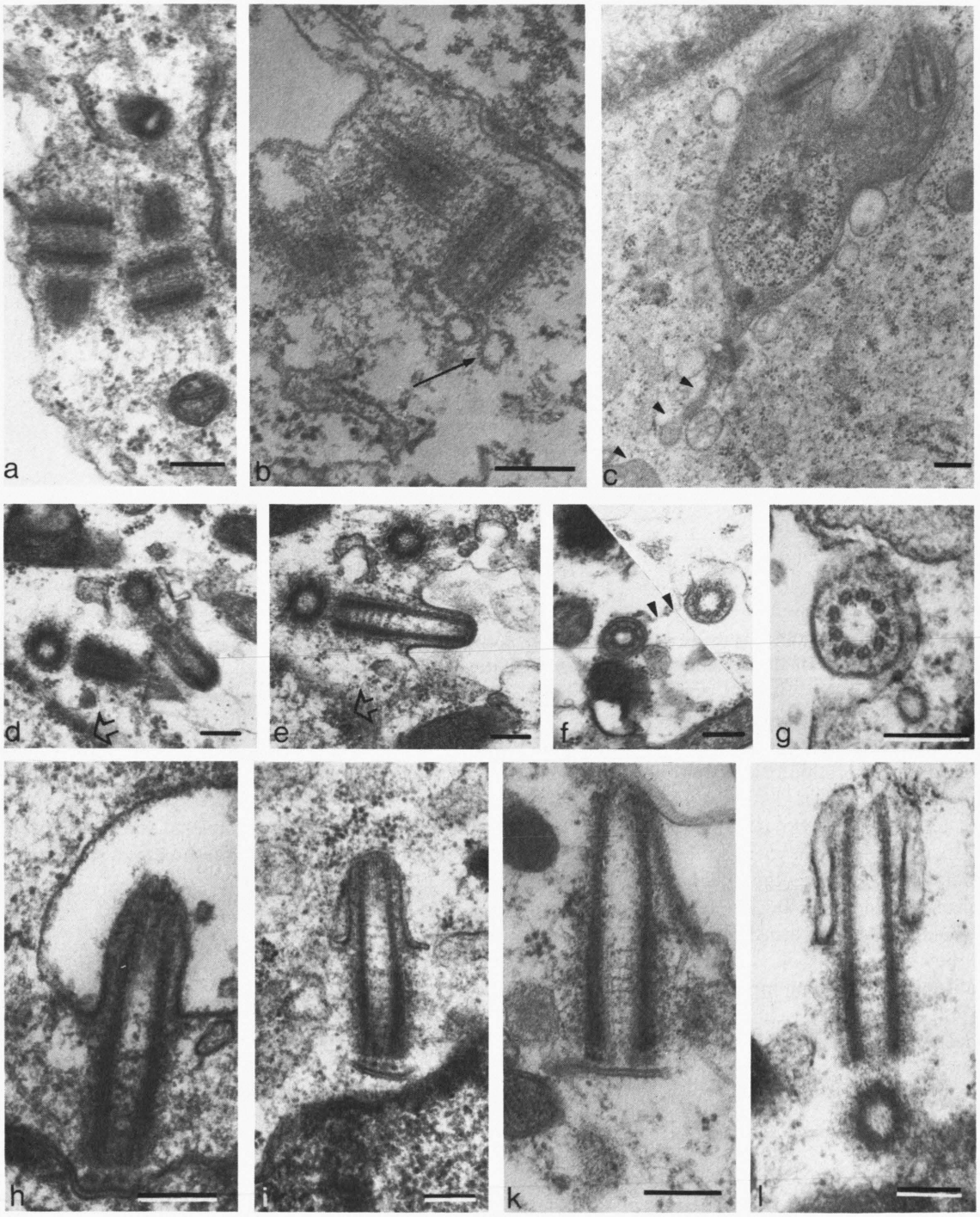


Fig. 1. The formation of primary cilia.
a–g) Origin of primary cilia in the centrosphere and h–l) detachment from the nuclear envelope.
a) The centriole cycle starts with a quadruple formation that consists of the symmetrically arranged parent centrioles and their daughter centrioles.
b) Spherical vesicles (arrow) associate with the distal ends of the centrioles and they later fuse to form an ampulla.

trioles carrying rudimentary cilia that protrude into a common cavity (Fig. 1d–f), enclosed by the ampulla's membrane. The primary cilia of *L. migratoria* spermatocytes extend the centriole (350 nm) to a maximum length of 1 μm. The diameter measures 200 nm at the ciliary base and 160 nm at the ciliary shaft. The space between the doublets seems to be empty, whereas the centriole (*i.e.* basal body) contains a series of regularly spaced, orthogonally arranged plates of fibrous material (Fig. 1e, h–l). Filaments also exist in the cytoplasm surrounding the rudimentary axoneme (Fig. 1g). Their arrangement may account for a striation of the ciliary membrane that is revealed by cutting the cilium tangentially (Fig. 2c).

In those early stages, the parent centriole of each diplosome is in contact with the nuclear envelope (NE), standing perpendicularly on the nucleus in a circular depression of its surface. In the space between the centriolar triplets and the NE, one can find globular electron-dense particles (Fig. 1h, i).

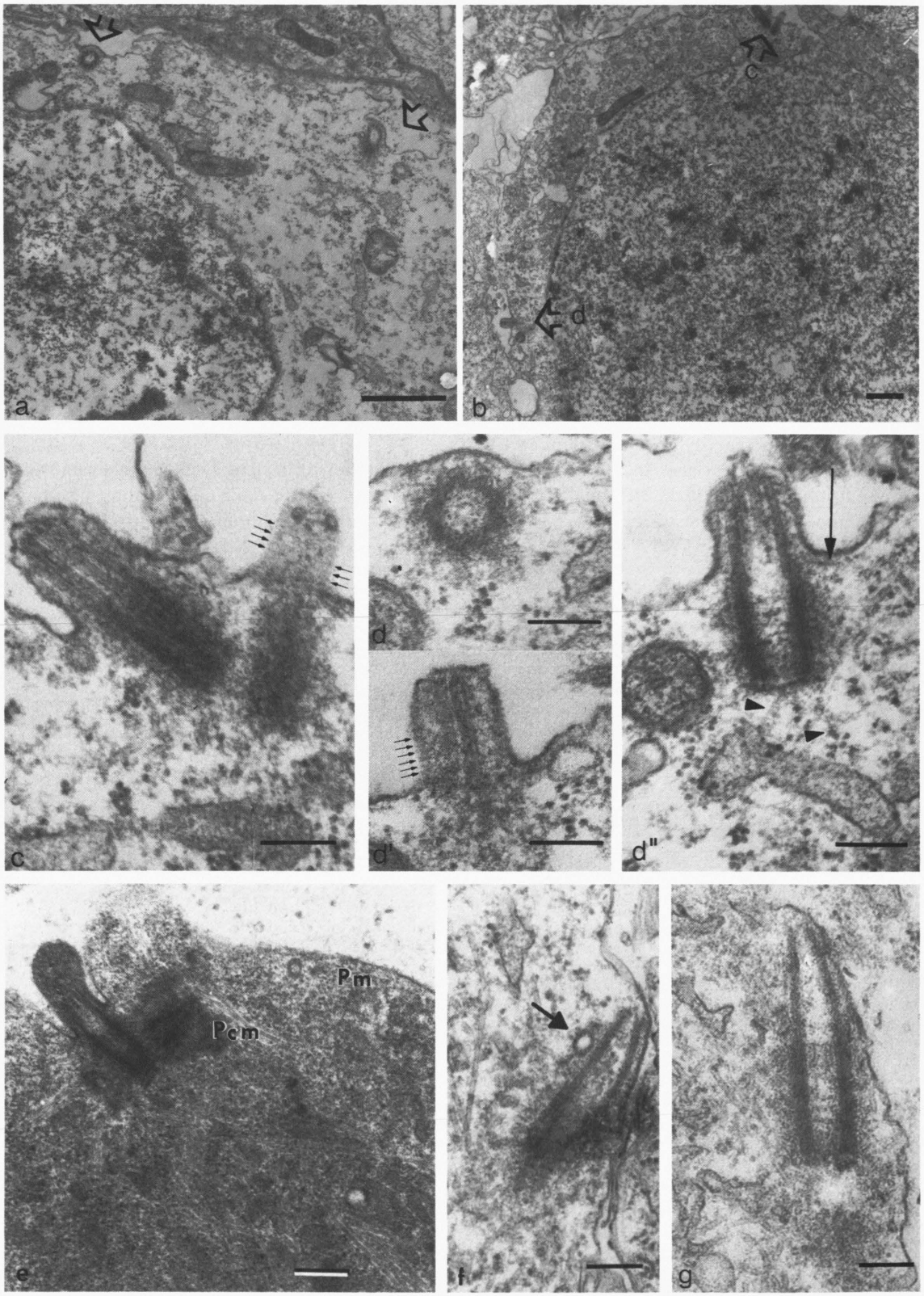
Subsequently, the membrane of the ampulla fuses with an invagination of the plasma membrane (arrowheads in Fig. 1c), thus making the ciliary membranes continuous with the plasma membrane. At that time, the formerly deep-seated ampulla is transfered to the cell surface so that finally the four primary cilia project into the extracellular space (Fig. 2a–c). Now the diplosomes are no longer in direct contact with the nucleus, but underneath the proximal ends of the centrioles one can occasionally find a flattened electron-dense vesicle that may well be derived from the nuclear envelope (NE). Fig. 1h–l show a series of electron-micrographs representing different stages of the diplosome-NE connection in a logical sequence.

Migration to the pole positions

The cells are still in late zygotene when the two diplosomes set out for their pole positions at opposite sides of the cell with all four centrioles staying

c) Longitudinally sectioned ampulla fuses with the invaginated plasma membrane (arrow-heads) to form a continuous membrane.
d–f) Photographs of a serially sectioned centrosphere; all four centrioles have extended primary cilia into the same cavity (arrows point to the nearby nuclear envelope); the two cross-sections in f) were taken from different section planes (arrow-heads point to the ampulla's membrane).
g) Cross-section of a primary cilium, demonstrating the (9 + 0)-organization of the rudimentary axoneme and the lack of dynein arms.
h) Original attachment to the nuclear envelope: Between the parent centriole in a circular depression of the nuclear envelope and the nucleus we find spherical electron-dense particles.
i) At the base of this slightly lifted primary cilium there is a collapsed vesicle.
k) This primary cilium, already extending into the extracellular space, has taken the vesicle along.
l) A daughter centriole, basally associated with the parent centriole, is also ciliated, but lacks a collapsed vesicle.
Bars: 200 nm.

Fig. 2. Ciliated centrosomes on their way to the pole positions and their fate in cell division.
a, b) Survey views of two prophases during centriole migration. In each cell, both centrosomes are visible since the direction of sectioning corresponds well with the direction of the centriolar movement. The arrows in a) and b) point at the diplosomes.
e–g) Primary cilia as parts of the centrosomes in the meiotic spindle.
a) Early state: The photograph shows two centrioles attached to the plasma membrane at a distance of ca. 3 μm. Serial sections of this cell demonstrated a totally ciliated diplosome at each position. These diplosomes have so far passed only one seventh of their way to the pole positions.
b) Advanced state: The separation of the diplosomes is half-finished. The letters c and d refer to the enlargements of the centrioles in c) and d).
c) The arrows indicate the ciliary striation.
d, d′, d″) Serial sections of the diplosome d in b) to show its two centrioles; in d′) arrows indicate the ciliary striation; d″) demonstrates most clearly the stiffening of the plasma membrane around the primary cilium (arrow) and the microfilaments at its base (arrow-heads).
e) A centrosome in metaphase: The centrioles are still ciliated; the collapsed vesicle at the distal tip of the centriole is no longer visible having disappeared in the pericentriolar material (Pcm). Pm, plasma membrane.
f, g) Centrosomes at metaphase/anaphase-transition: The primary cilia have turned by about 90 °C and lie underneath the cell surface.
Bars: a, b, 1 μm; c, d, f, g, 200 nm; e, 2 μm.


a
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c
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ciliated on their way (Fig. 2a–d). The plasma membrane surrounding each primary cilium is within a radius of 200 nm reinforced by additional material that forms a 10 nm thick sheath at the cytoplasmic side of the membrane at a distance of 115 nm (Fig. 2d″). Thus, the separating diplosomes are inserted in a stiffened part of the membrane by their rudimentary cilia and seem to be able to float in the plasma membrane like swimming islets in the sea. But since mere floatation would seem insufficient for a directed movement, we searched for something that could control or even drive this process. We found radially oriented microfilaments (mfs) at the basal tip of several centrioles (Fig. 2d″), some MTs which parallel both the plasma membrane and the NE, and similarly oriented ER cisternae. Ultrastructurally, there is no difference in the cytoplasm on either side of the separating diplosomes or in between them.

Fate of the primary cilia

These primary cilia persist throughout prophase and can still be found in late metaphase cells (Fig. 2e), where they appear somewhat retreated into a funnel-shaped cavity of the plasma membrane (pm). The densely stained lamellae at the base of the diplosomes are now obscured by electron-dense pericentriolar material (pcm, Fig. 2e), which has accumulated in the centriolar region during spindle formation. In anaphase cells, however, at least during the movement of the autosomes, we could never find protruding primary cilia (Fig. 2f, g). Obviously, the centrioles turn by 90° just at the metaphase/-anaphase transition, thus transferring the whole ciliary shaft underneath the cell surface. The plasma membrane just above the primary cilium still shows the characteristics of the ciliary membrane and on the cytoplasmic side of the primary cilium we often find a spherical vesicle whose membrane resembles the stiffened part of the former ciliary membrane. These vesicles are either closely associated with the proximal end of the ciliary shaft or, later in anaphase, are found in the surrounding cytoplasm. It is quite evident that this internalization of the primary cilia is a rapid process since we find the ciliary remnants already within the cytoplasm when the bivalents at the equatorial plate only show the very first indication of anaphasic chromosomal separation (white arrows in Fig. 3b). Concomitantly with this internalization, the withdrawl of the ciliary shaft takes place. This is demonstrated in Fig. 3a and b, showing the complete cilium in a longitudinal section shortly after plasma membrane detachment (Fig. 3a) and in a more reduced state within the cytoplasm at the spindle pole's position (Fig. 3b). Filamentous structures (black arrows in Fig. 3b) that are taken as an indication of an active migration adhere to the base of the centriole.

Discussion

Though not very familiar to many investigators of dividing cells, primary cilia [7] do occur frequently, and have been found up to now in nearly all embryonic tissues and in tissue cultures, *i.e.* in cells with a high mitotic index. Consequently they are absent in all blood cells, but surprisingly also in cancer cells. The role of these primary cilia in regulation of the cell cycle is now an active field of research. Most studies have indicated a strong association or correlation between the transient loss of a cilium and early mitogenic events (*e.g.* DNA-synthesis) [reviewed by 11].

Our observations concerning the formation of primary cilia in prophasic spermatocytes of *Locusta migratoria* support some previous findings in different mitotic cell types [reviewed by 18], since the basal association of vesicles, their fusion to form the ampulla, and the presence of cilia either in the centrosphere or at the cell periphery have already been shown by other investigators but refering to other cell types [rev. 11]. Exposure of primary cilia into the extracellular space as well as the ciliation of both centrioles of each centrosome, however, are very rare. Since the two-fold occurrence of primary cilia at one centrosome has only been shown for some other insect spermatocytes [19], this event may be characteristic of meiotic cells.

In addition, our investigations provide evidence regarding the precise time of "deciliation" which is still in question for insect spermatocytes and all other cells. There is only the agreement that this event takes place prior to the M-phase of the cell cycle and that cells become reciliated some time in the G_1-phase [20, 8–10]. The observed dislocation of the whole primary cilium to underneath the plasma membrane at the metaphase/anaphase-transition partly confirms the observations of Rieder *et al.* [8]. They noted a resorption of the primary cilium of mitotic cells, but it was shown to take place already

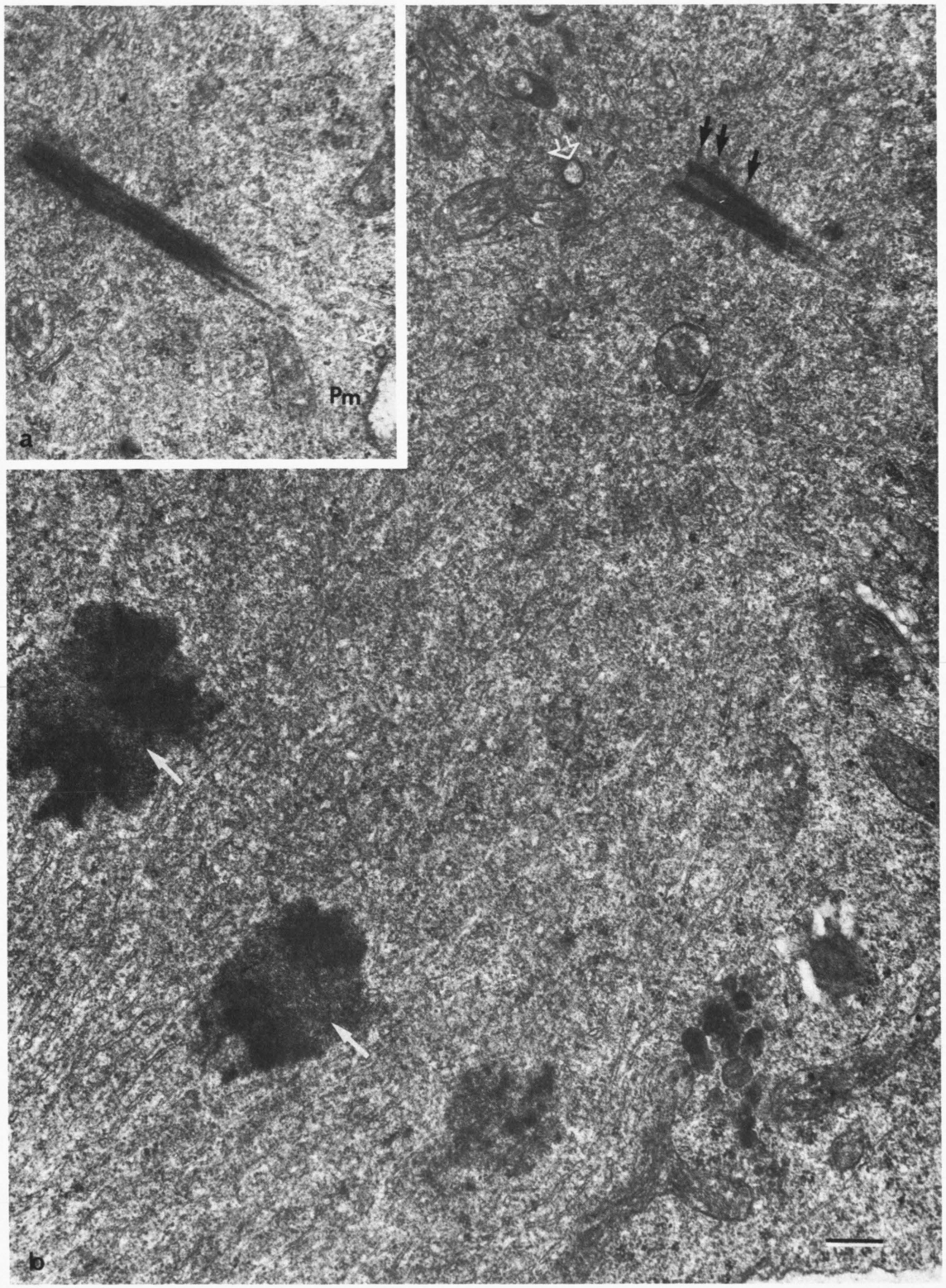

Fig. 3. Detachment of primary cilia from the plasma membrane (Pm) marks the onset of anaphase motion. Simultaneously, the ciliary shaft shortens: a) shows a primary cilium shortly after plasma membrane detachment; b) shows a more reduced state. Note spherical vesicles (open arrows) which presumably arise from the shiftened plasma membrane of the shaft and filamentous structures (black arrows) attached to the centriolar base. The white arrows point to the splitting bivalents of early anaphase. Bar: 2 μm.

in prometaphase and does not involve a positional change of the whole body. Interpreting their results, Rieder *et al.* [8] took the observed successive depolymerization of the doublets as an indication of a tubulin requirement for the growing spindle. This argument cannot be valid for the "deciliation" process in locust anaphases, since the dislocation of the ciliated centrioles to a position underneath the plasma membrane does not take place before the completion of the spindle apparatus.

The assumption of prophasic deciliation [11] seems to be indeed reasonable if one takes the separation of the centrioles from the plasma membrane as a necessary prerequisite for the migration of the centrosomes to the pole positions. However, just these movements can easily be explained by our EM-investigations if one assumes membrane fluidity and a contractility in the cell cortex that could involve the microfilaments (Mfs) registered at the base of the primary cilium which itself is tightly attached to the plasma membrane. The presence of actin in the cell cortex region has been demonstrated for locust spermatocytes of different stages by indirect immunofluorescence (IIF) [12, 13]. Recently, Euteneuer and Schliwa [21] also found strong evidence for an involvement of plasma membrane-bound actin filaments in the positioning and motility of centrosomes. Since we find no obvious structures only between the separating diplosomes in locust spermatocytes at later prophase, any pushing force to drive the centrosomes can be excluded. The observed MTs and ER cisternae, longitudinally paralleling their path, may, however, serve as a guiding principle, representing a railing to which the participating Mfs attach. The migration of the centrosomes would then not simply depend on a pushing force developed by the growing spindle [*e.g.* 22] which has already been rejected earlier [*e.g.* by Molè-Bajer, 23], nor is it achieved by a pulling force of the nuclear envelope [24].

According to a hypothesis of Tucker *et al.* [10], the most important physiological function of the extended primary cilia may be a sensory one, thus representing a receptor for extracellular stimuli. Poole *et al.* [25] recently supported this conception and thought of primary cilia in differentially developed connective tissue as cellular cybernetic probes which conduct certain extracellular stimuli to evoke cellular responses. In locust spermatocytes, the primary cilia could respond to any meiotic inductor in the extracellular medium.

In locust spermatocytes the exact time of "deciliation" seems to coincide with the separation of the bivalents, since primary cilia are present throughout the entire metaphase stage and could still be found during anaphase movement of the X-chromosome which precedes autosomal movement (unpublished observation), but they have withdrawn underneath the plasma membrane when the autosomes migrate. It appears to us possible that this change in the position of the whole centrosome triggers the separation of the bivalents by influencing the anchorage of the spindle fibres. A dependence of the succeeding chromosomes' movement on spindle fibre architecture has already been described by Sillers and Forer [26] for crane fly spermatocytes. In addition, a reduced tension in the no longer membrane-anchored spindle apparatus could transduce an extracellular stimulus to the kinetochores.

A very interesting clue to the rudimentary cilium's role in cell cycle controls comes from a Ca^{2+}-ionophore experiment [10] which shows "deciliation" as an indication of a preceding calcium flux. This Ca^{2+}-transport, which seems to be limited to the ciliary membrane [10], may be due to the array of particles, that accounts for the ciliary striation, being presumably similar to those found at the base of ciliary shafts of motile cilia [27]. A Ca^{2+}-flux from the extracellular medium to the cytoplasm could represent the initial Ca^{2+}-flux that was postulated by Hepler [28] as an extracellularly released trigger for anaphase onset, which causes an even larger intracellular Ca^{2+}-release that seems to regulate chromosomal movement. Thus, both an early Ca^{2+}-influx and the dislocation of the primary cilium seem to be initial steps of the cascade of events that has been postulated by Izant [5] for the regulation of anaphase, since he could stimulate a precocius onset of chromosomal movement by the injection of $CaCl_2$ but found no direct effect on the separation of the kinetochores.

Any regulatory step may also involve the flattened vesicle at the base of the centrosome which disappears during the accumulation of the pericentriolar material from prometaphase to metaphase. This putative remnant of the nuclear envelope seems, however, to be engaged in spindle formation rather than in triggering anaphase onset.

By demonstrating membrane-bound Ca^{2+} in locust spermatocytes with chlorotetracycline (CTC), we have found an intracellular Ca^{2+}-release prior to anaphasic chromosomal movement (unpublished re-

sults) and probably after the ciliary translocation, but further experiments on the exact point of time are needed to connect these results unambiguously. Then it should be possible to decide whether "deciliation" is connected with the triggering of kinetochore separation or chromosomal movement.

Acknowledgements

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