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Possible roles of actin and myosin during anaphase chromosome movements in locust spermatocytes

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Summary. We tested whether the mechanisms of chromosome movement during anaphase in locust (*Locusta migratoria* L.) spermatocytes might be similar to those described for crane-fly spermatocytes. Actin and myosin have been implicated in anaphase chromosome movements in crane-fly spermatocytes, as indicated by the effects of inhibitors and by the localisations of actin and myosin in spindles. In this study, we tested whether locust spermatocyte spindles also utilise actin and myosin, and whether actin is involved in microtubule flux. Living locust spermatocytes were treated with inhibitors of actin (latrunculin B and cytochalasin D), myosin (BDM), or myosin phosphorylation (Y-27632 and ML-7). We added drugs (individually) during anaphase. Actin inhibitors alter anaphase: chromosomes either completely stop moving, slow, or sometimes accelerate. The myosin inhibitor, BDM, also alters anaphase: in most cases, the chromosomes drastically slow or stop. ML-7, an inhibitor of MLCK, causes chromosomes to stop, slow, or sometimes accelerate, similar to actin inhibitors. Y-27632, an inhibitor of Rho-kinase, drastically slows or stops anaphase chromosome movements. The effects of the drugs on anaphase movement are reversible: most of the half-bivalents resumed movement at normal speed after these drugs were washed out. Actin and myosin were present in the spindles in locations consistent with their possible involvement in force production. Microtubule flux along kinetochore fibres is an actin-dependent process, since LatB completely removes or drastically reduces the gap in microtubule acetylation at the kinetochore. These results suggest that actin and myosin are involved in anaphase chromosome movements in locust spermatocytes.

Keywords: Chromosome movement; Mitosis; Actin; Myosin; Latrunculin B; 2,3-Butanedione monoxime; Myosin phosphorylation inhibitor.

Introduction

Nuclear division (karyokinesis or mitosis) includes reorganisation of different cell structures. As cell division begins, a bipolar spindle forms that ensures accurate segregation of the condensed chromosomes between daughter cells during anaphase. Most models proposed for chromosome movement consider anaphase as a microtubule-based mechanism, in which spindle microtubules and their motors are the key, if not the only, spindle components involved in the functioning of the spindle (reviewed in McIntosh et al. 2002) and in producing tubulin flux in kinetochore microtubules (Sawin and Mitchison 1991). Some models describe anaphase as an actin-based mechanism in which actin and myosin are involved in chromosome movement (Forer and Pickett-Heaps 1998, Silverman-Gavrila and Forer 2001, Silverman-Gavrila and Forer 2003) and work together in a spindle matrix (Fabian et al. 2007).

Actin and myosin have been found in a variety of spindles (Forer et al. 2003: table I, Margolin 2005) and various functional tests using inhibitors indicate that actin and myosin are needed for attachment of prometaphase chromosomes to the spindle in crane-fly spermatocytes (Forer and Pickett-Heaps 1998, Silverman-Gavrila and Forer 2000a), PtK cells (Sanger et al. 1989) and algae (Sampson et al. 1996), and that actin and myosin are required for anaphase movements in these and other systems (Schmit and Lambert 1990, LaFountain et al. 1992, Sampson et al. 1996, Forer and Pickett-Heaps 1998, Silverman-Gavrila and Forer 2001, Margolin 2005). Treatments of crane-fly spermatocytes in anaphase with actin or myosin inhibitors generally cause slowing or stopping of the movements of partner half-bivalents, though sometimes they have no effect (Silverman-Gavrila and Forer 2001, Fabian and Forer 2005). The chromosomes often recover in the continued presence of the drug. While these data implicate actin and myosin in anaphase chromosome movement, other components also may be utilised, since movements are normal

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in actin-free spindles formed by removing actin in prometaphase and keeping cells continuously in an anti-actin drug (Fabian and Forer 2005). Thus, redundant mechanisms may be present and available to move chromosomes during anaphase.

The localisations of various cytoskeletal proteins in spindles also suggest functional roles for actin and myosin. Confocal microscope studies of crane-fly spermatocytes, using immunocytochemistry, indicate that actin and myosin are located in spindles in positions where they would be expected to be, were they involved in force production (Silverman-Gavrila and Forer 2003). Another muscle protein, titin, responsible for muscle elasticity, also is present in crane-fly spermatocytes (Fabian et al. 2007), in association with the matrix proteins skeletor (Walker et al. 2000), megator (Qi et al. 2004), and chromator (Rath et al. 2004), consistent with the involvement of all these proteins with a spindle matrix (Fabian et al. 2007).

Actin and myosin also are required for tubulin flux within kinetochore microtubules in crane-fly spermatocytes (Silverman-Gavrila and Forer 2000b). Flux, a steady poleward movement of tubulin subunits in the kinetochore microtubules (Sawin and Mitchison 1991), is due to incorporation of tubulin at the kinetochore (polymerisation) coupled to depolymerisation of the microtubules at their minus ends at the pole (Mitchison 1989, Maddox et al. 2003). Tubulin flux is considered to be important for the poleward movement of chromosomes during anaphase (reviewed in Rogers et al. [2005]). Flux usually is measured after intracellular injection of fluorescent tubulin or photoactivable tubulin (e.g. Mitchison 1989, LaFountain et al. 2004). Other methods also have been used, e.g., staining with antibodies against total tubulin and against acetylated (stable) tubulin (Wilson et al. 1994, Wilson and Forer 1997, Forer and Wilson 2000). Kinetochore microtubules are more stable than the polar microtubules (discussed in Wilson and Forer [1989]) and these stable microtubules are acetylated (Palazzo et al. 2003). Staining with antibody against acetylated tubulin demonstrated acetylation only in the kinetochore microtubules. A "gap in acetylation" at the kinetochore (Wilson and Forer 1989) represents newly incorporated tubulin that has not had time to be acetylated. Thus, this gap is a measure of tubulin flux along kinetochore microtubules (Wilson et al. 1994, Wilson and Forer 1997). We studied flux in locust spermatocytes by measuring the gap in tubulin acetylation at the kinetochore.

Most of the data implicating actin and myosin in anaphase movements were obtained from crane-fly spermatocytes, which are unique in many ways. For example, sex chromosomes have fibres to both poles; they enter anaphase only after the autosomes reach the poles (Forer 1969); and there are signals between sex chromosomes (Ilagan and Forer 1997), between partner autosomes (Wong and Forer 2003), and between autosomes and sex chromosomes (Sillers and Forer 1981). Thus, we studied locust spermatocytes to see how general the conclusions drawn from crane-fly spermatocytes were. Locusts were available to us, and in many aspects of division, locust spermatocytes are similar to the better studied grasshopper spermatocytes, which have been used for a variety of experiments related to chromosome orientation in the spindle (Ault 1984, 1986; Rebollo and Arana 1995), microtubule attachment and role of tension in checkpoint signals (Nicklas et al. 2001), and anaphase movements of chromosomes (Ris 1949, Ault and Nicklas 1989, Paliulis and Nicklas 2000, Chen and Zhang 2004). We are aware of only a few papers that describe locust (*Locusta* spp.) spermatocytes. McClung (1902) and Mohr (1914) gave detailed descriptions of stages of division in spermatocytes of members of the family Locustidae on the basis of fixed and stained preparations. Rees and Jamieson (1954) described the supernumerary chromosome in *Locusta migratoria*. Moens (1969) described the fine structure of meiotic chromosome polarisation and pairing in *L. migratoria* spermatocytes. White (1935) studied the effects of X-rays on mitosis in *L. migratoria*. Using electron micrographs, Gawadi (1974) showed that actin filaments are present in locust spermatocyte spindles, that these filaments are parallel to the microtubules and that they occasionally attach to the chromosomes, most of them with the pointed end facing the equator. Other studies have shown that intracellular Ca^{2+} might have a role in controlling anaphase movements in locust spermatocytes by regulating microtubule assembly and disassembly, activation of actin filaments, or stimulation of a dynein-like ATPase (Beier and Hauser 1981). The role of actin in anaphase chromosome movements in locust spermatocytes was emphasised by studies using inhibitors (vanadate and taxol) which disrupt the spindle framework (Daub and Hauser 1986, 1988). These studies also support the model that considers microtubules not as the force producers in anaphase, but rather as the rate-limiting factor in anaphase motion, as originally proposed by Forer (1974). Since there have been few detailed descriptions of living spermatocytes, part of our study is a basic description of meiosis in living locust spermatocytes.

In addition to studying live control cells, we localised actin, myosin, and other potentially functional proteins in

spindles of locust spermatocytes, and we used actin inhibitors cytochalasin D (CD) and latrunculin B (LatB), myosin inhibitor 2,3-butanedione monoxime (BDM), and inhibitors of myosin phosphorylation Y-27632 ((*R*)-(+)-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate) and ML-7 (1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine) to test their involvement in anaphase chromosome movements and to determine whether actin is required for tubulin flux in kinetochore microtubules. We report the results in this article.

Materials and methods

Male 5th instar locusts (*Locusta migratoria*) were dissected under Halocarbon oil 200 (Halocarbon Products Corp., River Edge, N.J., U.S.A.) and testes were removed and put onto well slides under oil to prevent evaporation. Individual lobes were removed from the testis, rinsed with Belar's Ringer solution (Belar 1929) and placed in a fibrin clot in a perfusion chamber, following procedures similar to those used for crane flies (Fabian and Forer 2005, Forer and Pickett-Heaps 2005).

Living locust spermatocytes in perfusion chambers were kept in Belar's Ringer solution (Belar 1929) or treated at various times, as described in the text, with different inhibitors (1.5 or 2 μM LatB, 20 mM BDM, 20 or 30 μM CD, 50 μM Y-27632, 75 μM ML-7). Y-27632 and BDM were diluted about 1000-fold from stock solutions made up in Ringer solution; LatB, CD, and ML-7 were diluted about 1000-fold from stock solutions made up in dimethylsulfoxide (DMSO). DMSO at its final concentration of 0.1% has no effect on locust spermatocytes (Daub and Hauser 1988). This is similar to its lack of effect on crane-fly spermatocytes (at 0.2% DMSO [Forer and Pickett-Heaps 1998] and at 1% DMSO [LaFountain et al. 1992]) or on PtK cells (at 0.4% DMSO [Wrench and Snyder 2001]). The perfusion of Ringer solution itself had no effect on locust spermatocyte division either, similar to results for crane-fly spermatocytes (Forer and Pickett-Heaps 1998).

Images of live cells, taken by phase-contrast microscopy (Nikon oil immersion 100× objective, numerical aperture of 1.3), were recorded onto VHS tapes or directly onto DVDs. Time-lapsed images were obtained from VHS sequences by Adobe Premiere and from the real-time sequences on DVDs by Virtual Dub (http://fcchandler.home.comcast.net). Chromosome velocities were calculated by custom software and data were plotted by SlideWrite.

For immunostaining, we followed the protocol described in detail in Fabian and Forer (2005). We used the following solutions and antibodies: 2.2 M Alexa 488 phalloidin for filamentous actin (Molecular Probes), 1:200 My21 mouse immunoglobulin M antibody against myosin regulatory light chain (Sigma), 1:4000 YL1/2 rat antibody against tyrosinated tubulin (Kilmartin et al. 1982), 1:200 6-11B-1 mouse antibody against acetylated tubulin (Sigma), 1:500 mouse antibody against α-tubulin (Cedarlane), 1:500 α-KZ rat antibody against D-Titin (Machado et al. 1998), 1:100 mAb1A1 mouse immunoglobulin M antibody against skeletor (Walker et al. 2000), 1:100 6H11 mouse antibody against chromator (Rath et al. 2004), and 1:100 12F10 mouse antibody against megator (Qi et al. 2004). The fluorochromes we used were Alexa 488, Alexa 568, or Alexa 594 (Molecular Probes) conjugated with appropriate secondary antibodies. Images were obtained using an Olympus Fluoview 300 confocal microscope and were further processed by Image J (http://rsb.info.nih.gov/ij/) and Adobe Photoshop software.

Results

Control cells

Locust meiosis-I spermatocytes contain 11 pairs of telocentric autosomes of variable size and one large univalent sex chromosome, which does not align at the equator during metaphase but stays near one pole (Fig. 1) (McClung 1902, Rees and Jamieson 1954, John and Hewitt 1966). Prometaphase lasts for at least 120 min (at 20–25 °C), the longest we followed some of the cells. During prometaphase, the chromosomes move up and down in the spindle with variable speeds. Prometaphase ends when the autosomes are arranged in a metaphase plate, where they continually adjust their positions through small movements toward and away from the poles. The bigger autosomes are generally arranged at the exterior of the spindle, while the smaller ones are in the middle. On average, metaphase lasts 10 min (7–12 min), after which all autosomes enter anaphase at the same time and move with average constant separation velocities of 1.2 μm/min (Table 1), as calculated from graphs of interkinetochore

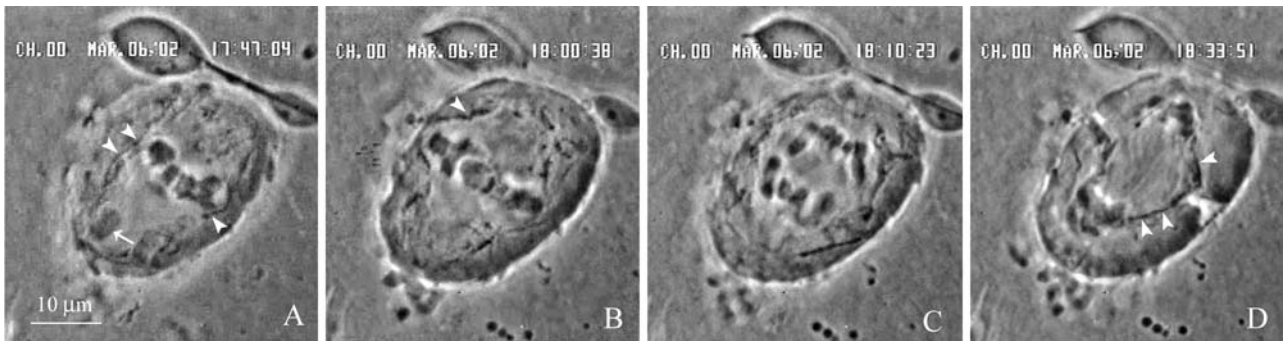

Fig. 1 A–D. Normal division in a locust spermatocyte. Phase-contrast images of prometaphase (**A**), metaphase (**B**), anaphase (**C**), and cytokinesis (**D**). The univalent sex chromosome (arrow in **A**) does not align at the equator but stays at one pole during division. The spindle area is outlined by mitochondria (arrowheads in **A**, **B**, and **D**). Bar: 10 μm

Table 1. Effect of inhibitors added in anaphase on chromosome movement

Treatment (nr. of cells)	Nr. of half-bivalent pairs followed:							Slowed movement		Accelerated movement	
	Total	Stopped	Slowed	Accelerated	Not affected	Recovered in drug	Slowed after washout	Avg (range) separation speed (μm/min)	Increment [a]	Avg (range) separation speed (μm/min)	Increment [b]
Control (16)	32									1.17 (0.59–2.31)	
BDM (7)	21	4	12	0	5	2	2	0.75 (0.33–1.18)	2.37		
Y-27632 (2)	8	2	5	0	1	6	0	0.65 (0.33–1.06)	2.1		
ML-7 (2)	7	1	4	1	1	0	2	0.98 (0.8–1.14)	1.42	1.36	1.79
LatB (7)	25	4	9	5	7	6	8	0.85 (0.37–1.84)	2.25	1.86 (1.18–2.62)	1.5
CD (2)	7	0	0	2	5					1.6 (1.35–1.85)	1.61

[a] Ratio of speed before treatment to that after treatment
[b] Ratio of speed after treatment to that before treatment

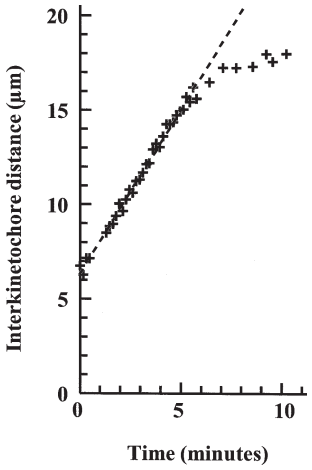

Fig. 2. Chromosome movement in a control cell, illustrating one pair of half-bivalents moving with constant separation velocity during anaphase. The dashed line represents the best fit (linear regression) through the indicated points

distance versus time for individual pairs of separating half-bivalents (Fig. 2). The different pairs of chromosomes reach the poles at about the same time, but because the interkinetochore distances at the start of anaphase are different and because those in the middle of the spindle travel distances different from those on the outside, the speeds of the different pairs in the cell vary somewhat. For all pairs, however, the pole-to-pole distance remains constant during the first part of anaphase. After the half-bivalents reach the poles, the spindle elongates, as previously described for locust spermatocytes by Beier and Hauser (1981). Because different chromosomes are in different focal planes, we could not follow all of the 11 pairs at the same time. We followed in detail only those in the plane of focus that we recorded, which limited us to 3–4 separating pairs per spermatocyte. Each set of points in our graphs is of one pair of separating half-bivalents, but we always followed more than that, invariably 3–4 pairs per cell.

Anaphase A is about 10 min long (range, 6–22 min), from the moment the bivalents disjoin until they reach the poles. Locust primary spermatocytes have a 20–24 μm long spindle (when flattened), while secondary spermatocytes have a slightly shorter spindle (16–20 μm). Though we did not follow primary spermatocytes after cleavage, meiosis II is reported to occur very rapidly after meiosis I with practically no interkinesis (White 1935). The spindle is outlined by mitochondria, which are distributed on both sides during the first division (Fig. 1), but only on one side during the second division.

Actin and myosin are present in locust spermatocytes

Actin filaments in locust spermatocytes were first described by Gawadi (1971, 1974), from electron micrographs. General staining of the spindle by immunofluorescence and actin antibodies was seen in nontreated locust spermatocytes by Beier and Hauser (1981). Our confocal microscope studies showed that actin is present both in the cortex and in the spindle during prometaphase, metaphase, and anaphase (Fig. 3), but it is organised differently than in crane-fly spindles. In locust spindles, actin is distributed in short filaments, in short stretches, in a finely dispersed

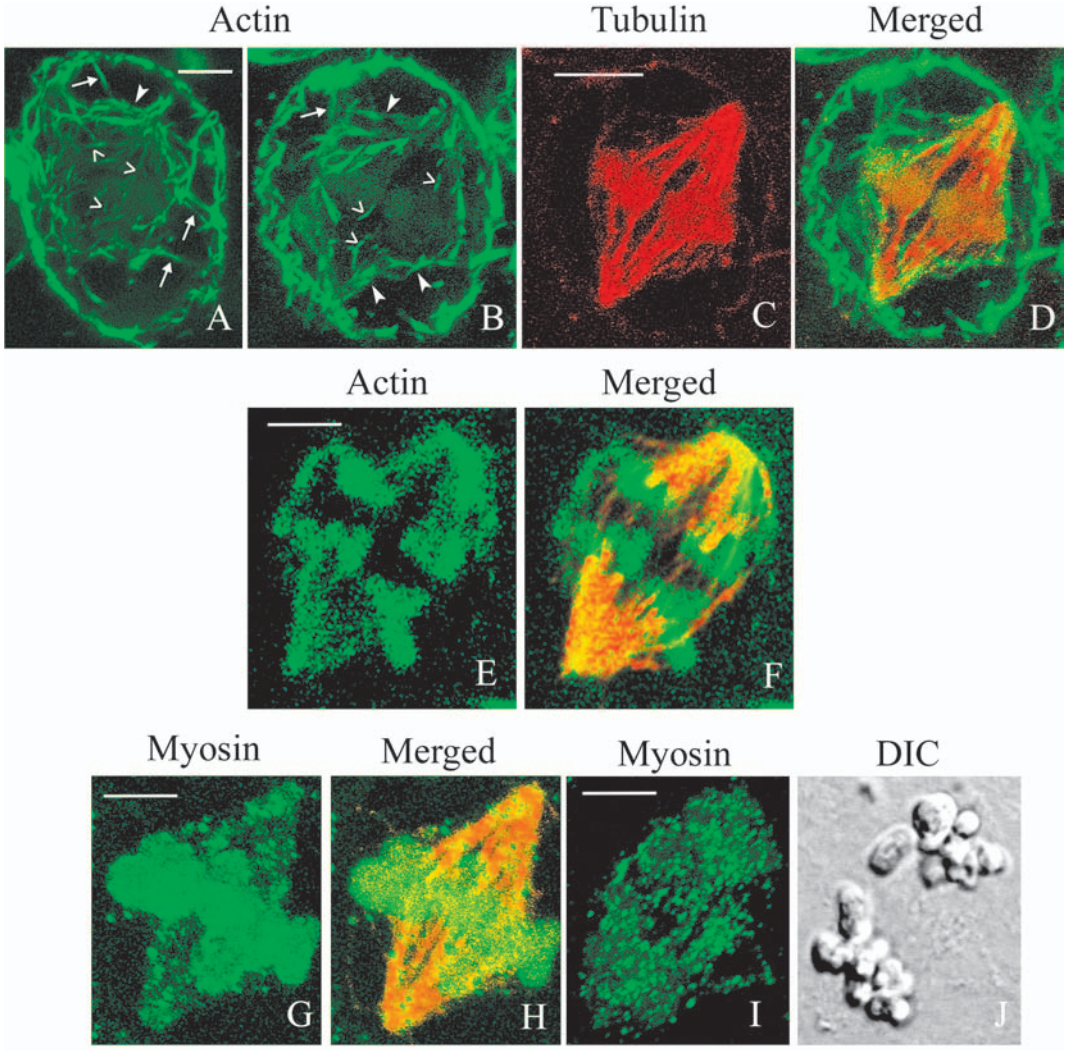

Fig. 3 A–J. Actin and myosin distribution in control locust spermatocytes. **A–F** Confocal microscope images of locust spermatocytes stained with Alexa phalloidin for filamentous actin (**A**, **B**, and **E**) and tubulin (**C**) and merged images of the two channels (**D** and **F**). **A** A cell in prometaphase; **B–D** a cell in metaphase; **E** and **F** a cell in anaphase. Actin forms a cage of filaments (closed arrowheads in **A** and **B**) around the spindle and the cage is attached to the cortex through several actin filaments (arrows in **A** and **B**). Spindle actin is present as stretches of short filaments (open arrowheads in **A** and **B**) and as a diffuse, finely punctate mass of actin. **G–I** Confocal microscope images of cells in metaphase (**G** and **H**) and anaphase (**I**) stained for myosin (**G** and **I**), and merged image of the myosin and actin channels (**H**). Yellow-orange in **D**, **F**, and **H** represents colocalisation of the two proteins. **J** Differential interference contrast image of the cell shown in **I**. Bars: 5 μm

punctate mass associated with the kinetochore and other microtubules, and as a compact (but weak) actin "area" in the spindle. The spindle is surrounded by many short actin filaments, forming a "spindle cage" which is distinct from the cortex. In places, this cage seems to send longer filaments of actin to the cortex, as if anchoring the spindle to the cortex (Fig. 3). The caged locust spindles appear similar to those described for many plant cells (e.g., Schmit and Lambert 1988, 1990; review in Staiger and Schliwa 1987). Actin colocalises with tubulin along the spindle fibres (Fig. 3). Myosin is present in the spindle and around the chromosomes in a punctate pattern, colocalised with tubulin along the kinetochore fibres (Fig. 3), as well as along polar microtubules and outside the microtubule fibres.

Several other proteins are present in the spindles of locust spermatocytes in addition to actin and myosin. In another work, we described in detail the distributions of titin, a muscle protein, and of several matrix proteins (skeletor, megator, chromator) in locust spermatocytes (Fabian et al. 2007). Here, for completeness, we briefly review their distribution. Titin is present in the spindle, in the chromosomes, at the poles, and in a punctate distribution along kinetochore fibres (Fig. 4). Skeletor is present in the spindle and at the poles; it colocalises with tubulin

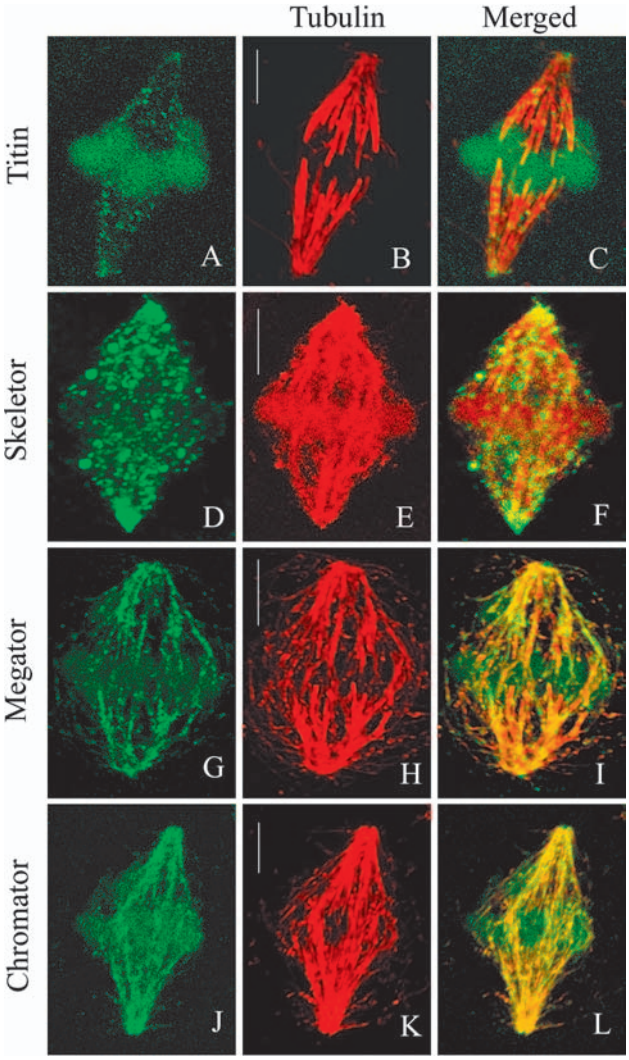

Fig. 4 A–L. Distribution of titin and matrix proteins in control locust spermatocytes. Confocal microscope images of cells in metaphase stained with antibodies against titin (**A**), skeletor (**D**), megator (**G**), chromator (**J**), and tubulin (**B**, **E**, **H**, and **K**). **C**, **F**, **I**, and **L** Merged images of the two channels. Yellow-orange in **C**, **F**, **I**, and **L** represents colocalisation of the proteins. Bars: 5 μm

along spindle fibres (Fig. 4). Megator and chromator have a dotted appearance and they are closely associated with microtubules during all phases of division (Fig. 4). They are present in the chromosomes, in the nuclear membrane, in the spindle, along all microtubules (kinetochore, polar, astral microtubules), and in the midbody, but not in the contractile ring. Megator, chromator, and titin are closely associated with actin and myosin in chromosomes, at the poles, and along nonkinetochore and kinetochore microtubules, but they are organised differently and do not always colocalise (Fabian et al. 2007). Thus, these muscle and matrix proteins are present in locust spermatocytes in locations at which they could be involved in chromosome

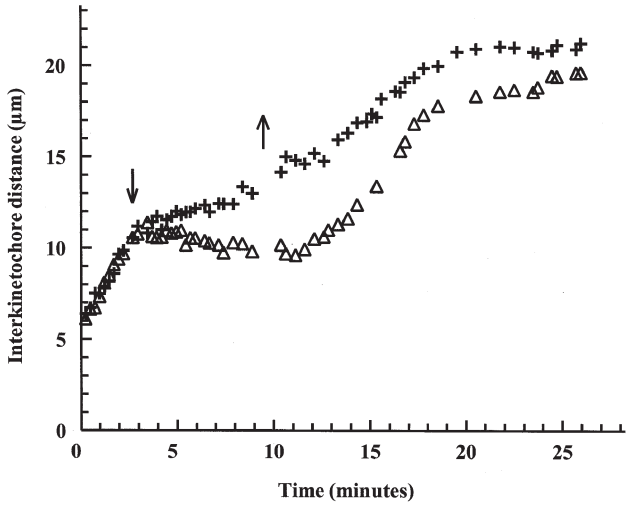

Fig. 5. Effects of BDM on chromosome movement in a locust spermatocyte when added during anaphase. Two pairs of half-bivalents from the same cell are shown (crosses and triangles). BDM was added after anaphase had started (down-pointing arrow) and was washed out after 7 min (up-pointing arrow). One pair of half-bivalents stopped (triangles) and the other drastically slowed (crosses). The stopped pair resumed movement at normal speed after washout

movement, perhaps functioning together in a contractile spindle matrix (Fabian et al. 2007).

After studying the localisations of actin, myosin, and associated proteins in locust spindles, we studied the physiological roles of actin and myosin in these spermatocytes by using various actin and myosin inhibitors.

Effects of myosin inhibitors and myosin-activation inhibitors on chromosome movements

To test for myosin involvement in anaphase chromosome motion, we treated anaphase cells with BDM, ML-7, and Y-27632. The drugs were added several minutes after disjunction of the bivalents, after the partner half-bivalents had moved sufficiently poleward to determine (post hoc) their rate of movement but early enough so that we could detect (post hoc) effects of the drugs on movement.

BDM inhibits motility by acting directly on myosin (review in Fabian and Forer [2005] and Forer and Fabian [2005]). When added to locust spermatocytes in anaphase, it generally slowed or stopped chromosome movement (Table 1). For this and other inhibitors, different chromosome pairs in the same cell often were affected differently. For example, in Fig. 5, which illustrates two different separating half-bivalent pairs in the same cell, BDM caused one pair to move more slowly and the other pair to stop.

Myosin also can be inhibited indirectly, by blocking its phosphorylation. Myosin is activated by phosphorylation

of its regulatory light chain mainly through myosin light chain kinase (MLCK) or Rho-kinase (Rho-K) (review in Matsumura [2005]). MLCK and Rho-K, which have been shown to have different spatial roles in phosphorylating myosin (Totsukawa et al. 2000, 2004; Katoh et al. 2001; Niggli et al. 2006), can be inhibited by ML-7 (Saitoh et al. 1987) and Y-27632, respectively (Narumiya et al. 2000, Ishizaki et al. 2000). ML-7 (Fig. 6) or Y-27632 (Fig. 7) added to anaphase locust spermatocytes stopped, slowed, or rarely accelerated (ML-7 only) chromosome movements (Table 1 and Fig. 6). (As a result of the small numbers of pairs studied, we cannot be certain whether or not the absence of acceleration with Y-27632 represents a real difference from ML-7.) These drugs were about as effective as BDM at slowing or stopping chromosome movement (Table 1). Therefore, these results support the previous ones obtained with BDM, implicating myosin in anaphase chromosome movements. The inhibition of chromosome movement in locust spindles by both ML-7 and Y-27632 suggests that nonmuscle myosin activity in mitosis in locust cells might be controlled through both Rho-K and MLCK, unlike in crane-fly spermatocytes, where ML-7 did not stop or slow chromosome movements (Fabian and Forer 2005).

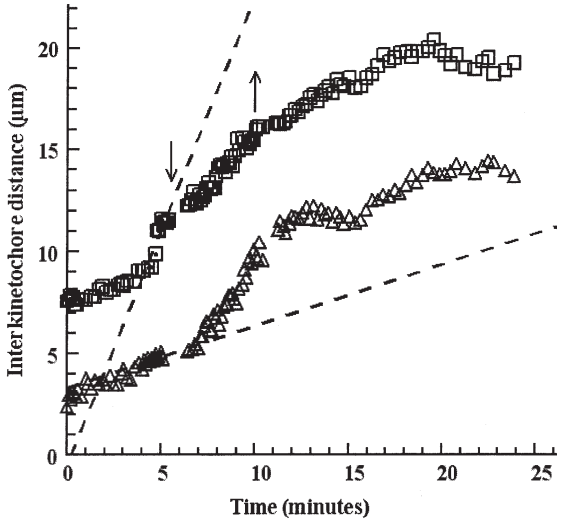

Fig. 6. Effect of ML-7 on chromosome movement in a locust spermatocyte when added during anaphase. Two pairs of half-bivalents from the same cell are shown. One pair slowed (squares) and one pair accelerated (triangles) after addition of ML-7 (down-pointing arrow). Sometimes, movement became slower after ML-7 was washed out (up-pointing arrow). The lines represent the best fit (linear regression) through the indicated points before addition of ML-7

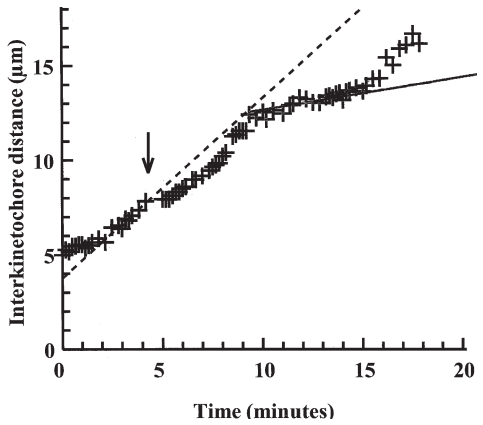

Fig. 7. Effect of Y-27632 on chromosome movement in a locust spermatocyte when added during anaphase. One pair of half-bivalents is shown (crosses), which slowed about 5 min after addition of Y-27632 (down-pointing arrow) and then partially recovered while still in the drug. The effect of Y-27632 is visible only after some minutes because of the drug's slow permeation (Narumiya et al. 2000). The lines represent the best fit (linear regression) through the indicated points. The dashed line is before and the solid line is after addition of Y-27632

For both BDM and Y-27632, chromosome movement sometimes recovered while cells still were immersed in the drug (Table 1). Since the inhibitors remained in the solution bathing the cells, which periodically was refreshed, it seems unlikely that recovery is due to metabolic degradation of the intracellular inhibitor, but rather to the cell being able to circumvent the blocked myosin.

Effects of actin inhibitors on chromosome movements

To test for possible roles of actin in chromosome movement, we treated anaphase cells with the actin inhibitors CD and LatB. CD is an inhibitor of actin polymerisation (Cooper 1987, Sampath and Pollard 1991); its effects on

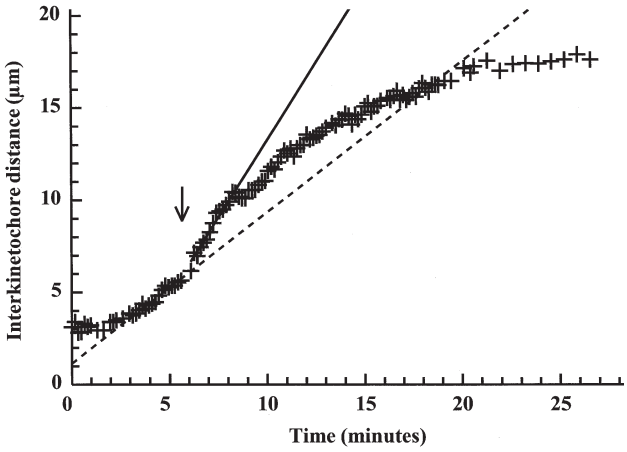

Fig. 8. Effect of CD on chromosome movement in a locust spermatocyte when added during anaphase. One pair of half-bivalents is shown (crosses). Chromosomes accelerated after addition of CD (down-pointing arrow) and then slowed while in the presence of the drug. The lines represent the best fit (linear regression) through the indicated points. The dashed line is before and the solid line is after addition of CD

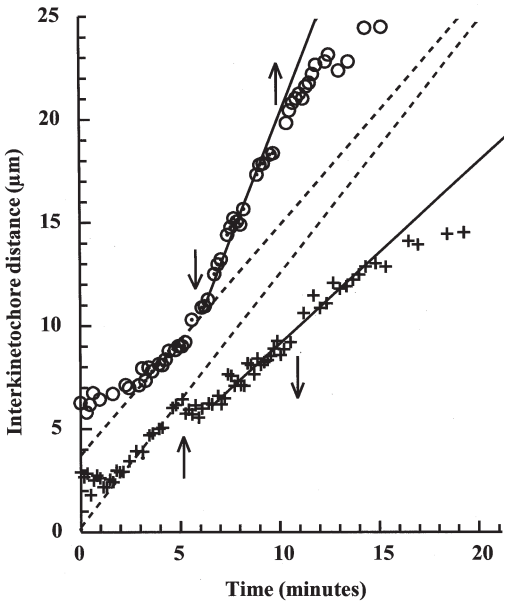

Fig. 9. Effects of LatB on chromosome movement in locust spermatocytes when added during anaphase. Two pairs of half-bivalents from different cells are shown (crosses and circles). LatB was added at 5 or 6 min, respectively, after anaphase started (the two leftmost arrows) and was washed out after 6 or 4 min, respectively (the two rightmost arrows). One pair of half-bivalents stopped for few minutes and then started again, at a very slow speed (crosses), while the other pair accelerated after addition of LatB (circles). The dashed lines are before and the solid lines are after addition of LatB

various types of cells and motile processes are reviewed in Forer and Pickett-Heaps (1998). LatB depolymerises actin filaments and binds to actin monomers (Spector et al. 1983, Coue et al. 1987, Ayscough 1998, Yarmola et al. 2000, Morton et al. 2000). When added during anaphase, CD had little effect on chromosome movements in locust spermatocytes (Table 1 and Fig. 8), while LatB generally slowed, stopped, or accelerated chromosome movements (Table 1 and Fig. 9). There were similar differences between CD and LatB when added earlier. CD added during metaphase (2 cells), 1–14 min before anaphase onset, had no effect on chromosome movement in subsequent anaphase. However, LatB added during metaphase (2 cells), 6–13 min before anaphase, disturbed chromosome movement during the subsequent anaphase; movement speeds were not constant throughout anaphase, but were biphasic. In the initial phase, some of the chromosomes moved slightly slower than normal or in the lower range of normal values, while in the second phase, all the chromosomes moved with slower than normal speeds (0.25–0.4 μm/min, compared to 0.59–2.13 μm/min in control cells). When LatB was added during prometaphase (1 cell), 25 min before anaphase, the chromosomes disjoined very slowly and they failed to finish anaphase. These results indicate that actin is required for normal anaphase in locust spermatocytes.

LatB added in metaphase, and in some cases when added in anaphase, also seemed to affect how far apart the two half-bivalents separated, in that they did not move as far apart as in control cells. The total distance the chromosomes moved in treated cells (15–16 μm) was less than the shortest distance we recorded in control cells (17 μm).

To test whether actin was absent from the treated spindles, we stained some of the preparations with phalloidin.

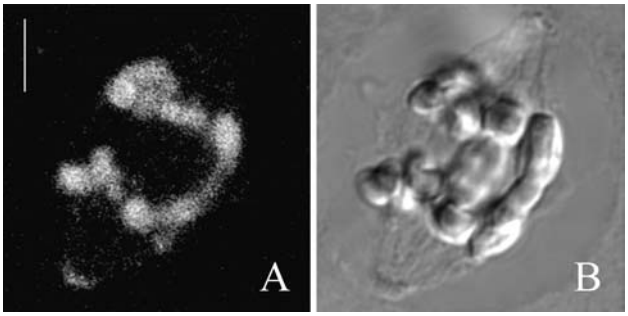

Fig. 10 A, B. Actin filaments are completely depolymerised after treatment of locust spermatocytes with LatB for 5 min. **A** Confocal microscope image of a cell in anaphase stained with Alexa phalloidin for filamentous actin; **B** differential interference contrast image of the same cell illustrating the chromosomes and the spindle fibres. Phalloidin stained the chromosomes in this cell, which also sometimes occurs in control cells, as it does in cells from other species, such as crane-fly spermatocytes (e.g., Silverman-Gavrila and Forer 2000a, Forer et al. 2003). Bars: 5 μm

Table 2. Effects of actin and myosin inhibitors on chromosome movement in crane-fly and locust spermatocytes

Inhibitor, mitotic stage of addition	Effects on anaphase chromosome movements in spermatocytes of:	
	Crane fly	Locust
LatB, anaphase	stop; slow; no effect[a,b]	slow; stop; accelerate; no effect
LatB, metaphase	slow; stop; variable speeds[a]	variable speeds
LatB, late prometaphase	slow; stop; variable speeds[a,b]	disjoin slowly; slow and anaphase not completed
CD, anaphase	stop; slow; no effect[a]	no effect; accelerate
CD, metaphase	disjoin and stop; slow; variable speeds[a]	no effect
BDM, anaphase	stop; slow; no effect[b,c]	slow; stop; no effect
ML-7, anaphase	no effect; accelerate[b]	slow; stop; accelerate
Y-27632, anaphase	stop; slow[b]	stop; slow

[a] Forer and Pickett-Heaps (1998)
[b] Fabian and Forer (2005)
[c] Silverman-Gavrila and Forer (2001)

In cells treated with LatB for 5–10 min, filamentous actin was totally or almost completely absent from the spindle (Fig. 10). Thus, LatB treatment caused loss of spindle actin, as in other cells (Fabian and Forer 2005).

All the inhibitors we used had reversible effects on chromosome movement. After they were removed from the preparations, the chromosomes generally resumed their initial speeds, they finished anaphase and the cells underwent cytokinesis. Sometimes, however, chromosome movement slowed after washing out the drug (Table 1), as also described for crane-fly spermatocytes treated with LatB or BDM (Fabian and Forer 2005).

There are several differences between crane-fly and locust spermatocytes (Table 2). One is the acceleration of chromosome movement: CD, LatB, and ML-7 sometimes caused acceleration (by 50–80%) of chromosome movement during anaphase in locust spermatocytes (Table 1), but CD and LatB never caused acceleration in crane-fly spermatocytes. Another difference is that CD added in anaphase or in metaphase does not block chromosome movement in locust spermatocytes (Table 1) but it does block it in crane-fly spermatocytes (Table 2), an effect which may be due to the different organisation of spindle actin in the two cell types.

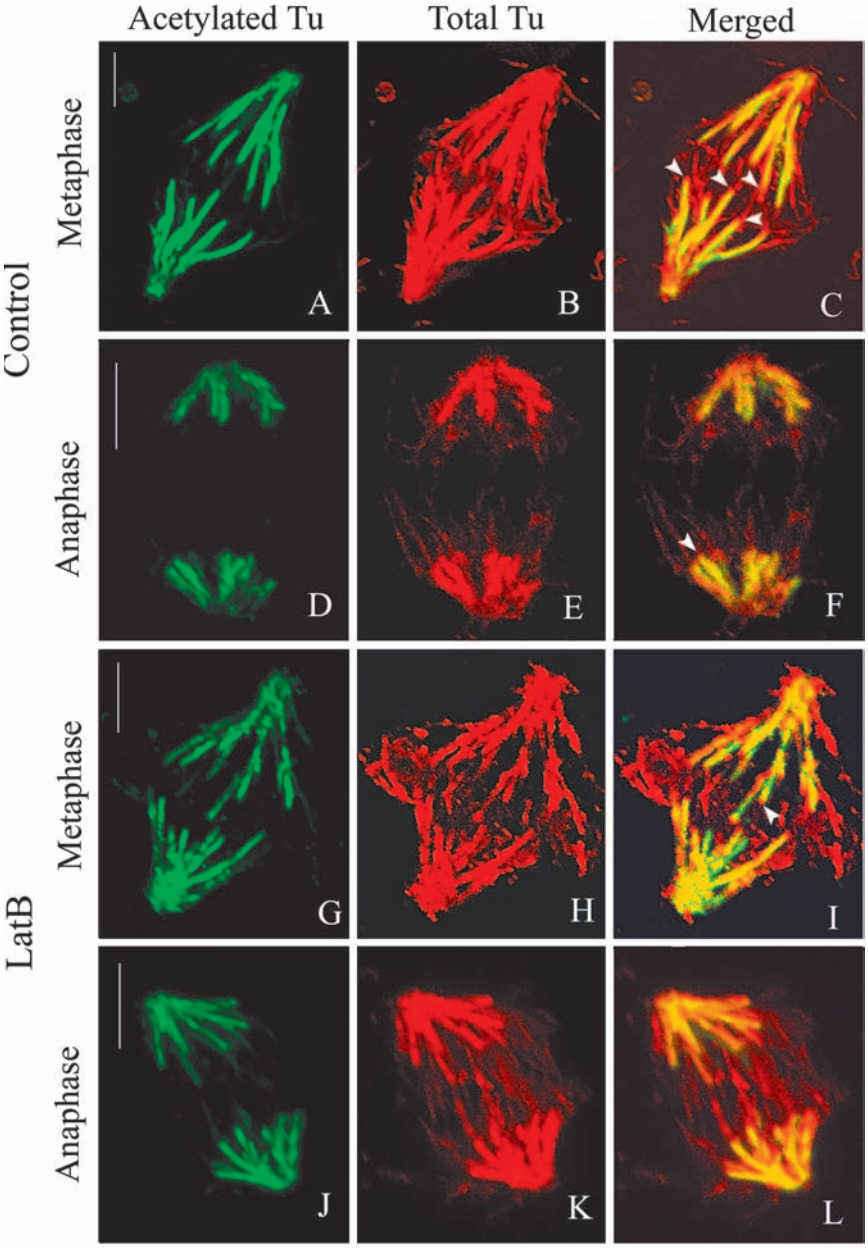

Fig. 11. Confocal microscope images of locust spermatocytes in metaphase (**A–C** and **G–I**) and anaphase (**D–F** and **J–L**) stained for acetylated tubulin (green) and tyrosinated tubulin (red). Yellow-orange in merged images (**C**, **F**, **I**, and **L**) represents colocalisation of the two proteins. Tubulin is acetylated along the kinetochore spindle fibres, except for a short (red) region near the kinetochores, which forms a gap in acetylation (arrowheads). The gap is much smaller in anaphase (**D–F**) than in metaphase (**A–C**). Treatment with LatB reduces the gap, both in metaphase (**G–I**) and in anaphase (**J–L**). Bars: 5 μm

Effects of inhibitors on cytokinesis

Cytokinesis in control locust spermatocytes lasts about 15 min. We did not directly study the effect of various inhibitors on cytokinesis, but we observed that the actin inhibitors (LatB and CD) and the Rho-K inhibitor (Y-27632) blocked furrow initiation and cytokinesis when added during metaphase or anaphase, regardless of whether the drug was removed or not by the time cytokinesis should have started. The MLCK inhibitor (ML-7) did not block cytokinesis initiation and completion when it was removed from the preparations before the start of cytokinesis.

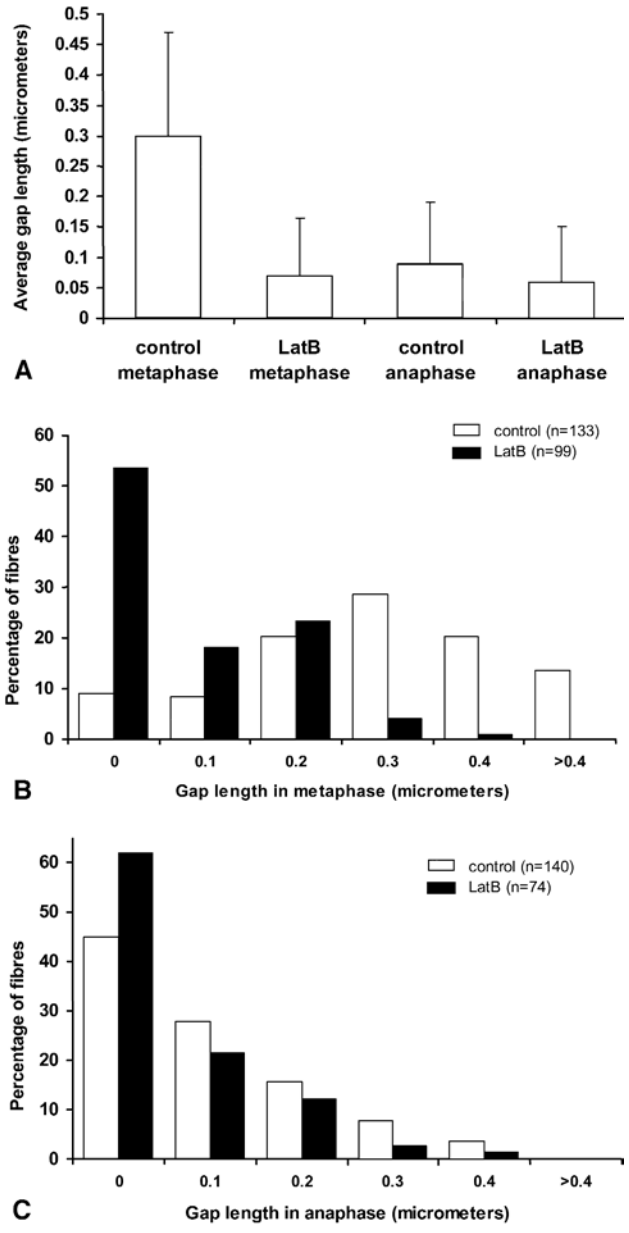

Fig. 12. A Length of the gap in acetylation at the kinetochores in control and LatB-treated locust spermatocytes. **B** and **C** The presence of a gap in metaphase and anaphase in control locust spermatocytes indicates that there is poleward flux along kinetochore microtubules; the drastically reduced gap in LatB-treated locust spermatocytes indicates that actin has a role in the poleward tubulin flux in locust cells

Effects of actin inhibitors on poleward tubulin flux

Previous studies of crane-fly spermatocytes showed that actin and myosin are involved in poleward tubulin flux (Silverman-Gavrila and Forer 2000b), so we tested this in locust spermatocytes. We stained locust preparations for acetylated tubulin (stable) versus tyrosinated tubulin (total tubulin). The gap in acetylation of the kinetochore microtubules at the kinetochores (Fig. 11), which on average was 0.3 μm long (Fig. 12), indicates that there is flux along kinetochore microtubules in normal (control) metaphase locust spermatocytes (discussion in Wilson et al. [1994]). In late-anaphase control cells, the gap was considerably smaller than in metaphase cells, which indicates that tubulin flux continues during anaphase, but by late anaphase it is reduced relative to metaphase (Fig. 11). To test if actin is required for tubulin flux in locust spermatocytes, we treated the cells with LatB to depolymerise actin and then stained for acetylated versus tyrosinated tubulin. When actin was depolymerised the gap in acetylation along kinetochore microtubules at metaphase was drastically reduced (Fig. 12) and was completely abolished in many of the cells. In anaphase cells, the gap in acetylation also decreased after treatment with LatB (Fig. 12). These results suggest that tubulin flux along kinetochore microtubules is an actin-dependent process in locust spermatocytes, similar to that in crane-fly spermatocytes.

Discussion

We studied actin and myosin involvement in chromosome movements in locust spermatocytes because much of the previous evidence for their participation in anaphase movements was obtained from experiments using spermatocytes from a single species, crane-fly, and we wanted to see how general the phenomenon was. Our data implicate actin and myosin in anaphase chromosome movements in locust spermatocytes, similar to crane-fly spermatocytes, but there are some differences in the details of their organisation and function.

Actin and myosin are present in locust spermatocytes in positions and with patterns of distribution that are consistent with them having a role in anaphase chromosome movement. The presence of actin in locust spermatocyte

spindles has been documented previously by Gawadi (1974) in electron micrographs, using heavy meromyosin binding to actin filaments, by Beier and Hauser (1981) after immunostaining nontreated cells with actin antibodies, and by Daub and Hauser (1986) after immunostaining vanadate-treated locust cells with actin antibodies. Using confocal microscopy of phalloidin-stained locust spermatocytes, we found short actin filaments and punctate actin in the spindle, associated with spindle microtubules, and we found longer actin filaments in a strongly stained cage of filaments around the spindle. In crane-fly spermatocytes, on the other hand, there is no such cage and the actin filaments in the spindle are longer and associated primarily with the kinetochore microtubules.

With respect to myosin, we know of no previous reports of myosin being present in locust spermatocyte spindles. Through confocal microscope studies, we showed that myosin is present along the spindle fibres (kinetochore and nonkinetochore ones), where it colocalises with tubulin, similar to its localisation in crane-fly spermatocytes.

Chromosome movements in locust spermatocytes were affected by actin and myosin inhibitors, but the effects were somewhat different from those in crane-fly spermatocytes. All of the inhibitors we used altered chromosome movements during anaphase but they showed a larger spectrum of effects than in crane-fly spermatocytes (Table 2). The two major differences between locust and crane-fly spermatocytes are in the effects of ML-7 and CD. In crane-fly spermatocytes, ML-7 either has no effect on movement or it causes acceleration (Fabian and Forer 2005), whereas in locust spermatocytes, ML-7 generally causes stopping or slowing and rarely acceleration or has no effect (Table 1). This might be because in crane-fly spermatocytes, spindle myosin is activated primarily by Rho-kinase, whereas in locust spermatocytes, myosin is phosphorylated almost equally by the two kinases (Rho-kinase and MLCK). This interpretation is consistent with results obtained with other cellular systems in which Rho-kinase is more active at the cell centre, while MLCK is more active at the cell periphery (Totsukawa et al. 2004, Matsumura 2005). Also, it is in agreement with the more rapid recovery (while in the presence of the drug) of the majority of the half-bivalents in locust spermatocytes treated with Y-27632 compared with those in crane-fly spermatocytes. This suggests that backup mechanisms are available for the cell to overcome interference with anaphase and that these mechanisms are more easily and readily turned on.

In locust spermatocytes, the only effect of CD added in anaphase or metaphase was to accelerate some of the chromosomes (Table 1), whereas in crane-fly spermatocytes, this generally slowed or stopped chromosome movement (Forer and Pickett-Heaps 1998, Fabian and Forer 2005). Thus, actin would seem to function in locust spermatocytes differently than in crane-fly spermatocytes. These differences in the effects of CD in the two cell types might be due to the different organisation of actin, with short stretches of actin in the locust spindle versus long actin filaments in crane-fly spindles. Also, the presence of an actin cage around the spindle in locust spermatocytes, similar to plant spindles (Staiger and Schliwa 1987), might interfere with CD action. The similar effects of LatB in slowing or stopping chromosome movements in both locust and crane-fly spermatocytes (Table 2) confirm that actin is involved in anaphase chromosome movements in these two cell types. The stopping or slowing effects of LatB added in anaphase are less severe in locust spermatocytes than in crane-fly spermatocytes, while those of LatB added in metaphase are more severe (Table 2), which may indicate that some spindle components are organised in a different temporal manner, or that there are additional alternate ways to move chromosomes to the pole in locust spermatocytes.

There are some similarities between the two cell types. For example, actin and myosin seem to be involved in producing flux along kinetochore microtubules. In locust spermatocytes, LatB blocks, or drastically reduces, the poleward flux of tubulin in both metaphase and anaphase cells. Metaphase flux in crane-fly spermatocytes is inhibited similarly by anti-actin drugs and also by anti-myosin drugs (Silverman-Gavrila and Forer 2000b). Thus, in both cell types, tubulin flux along kinetochore microtubules is driven, at least in part, by actin and myosin. Our experiments on locust spermatocytes indicate that flux, as measured by the gap in acetylation, continues to late anaphase; results which are consistent with measurements of flux in grasshopper (Chen and Zhang 2004) and crane-fly spermatocytes (LaFountain et al. 2004).

Another similarity between locust and crane-fly spermatocytes is that different chromosome pairs in a cell do not respond in the same way to a specific treatment (Forer and Pickett-Heaps 1998, Wong and Forer 2003, Fabian and Forer 2005). In locust spermatocytes, some of the slowed or stopped half-bivalents recovered while still in the presence of the inhibitor, mainly in the cells treated with LatB and Y-27632 (Table 1), as did some of the chromosomes in crane-fly spermatocytes (Forer and Pickett-Heaps 1998, Fabian and Forer 2005). A further similarity is that the chromosome movements in both species sometimes became slower after removing the drug (Table 1)

(Fabian and Forer 2005). These results are consistent with the notion that there are alternative ways in which the kinetochore fibres function, even in the same spindle (Fabian and Forer 2005, Cameron et al. 2006), and that when one mechanism of movement is inhibited or interfered with, another mechanism is subsequently activated to move the chromosomes poleward.

In summary, actin, myosin, and other cytoskeletal proteins are present in locust spermatocytes and the effects of inhibitors indicate a role for actin and myosin in anaphase chromosome movements and in flux in locust spindles.

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