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Research Article

Disruption of sperm release from insect testes by cytochalasin and β-actin mRNA mediated interference

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Abstract. Release of sperm bundles from moth testes is controlled by the local circadian oscillator. The mechanism which restricts migration of sperm bundles to a few hours each day is not understood. We demonstrate that a daily cycle of sperm release is initiated by the migration of folded apyrene sperm bundles through a cellular barrier at the testis base. These bundles have conspicuous concentrations of actin filaments at their proximal end. Inhibition of actin polymerization by cytochalasin at a specific time of day inhibited sperm release from the testis. Likewise, application of double-stranded actin RNA specifically inhibited sperm release. This RNA-mediated interference (RNAi) lowered the pool of actin mRNA in tissues involved in sperm release. The decline in mRNA levels resulted in the selective depletion of F-actin from the tip of apyrene sperm bundles, suggesting that this actin may be involved in the initiation of sperm release. Combined results of RNAi experiments at physiological, cellular and molecular levels identified unique cells that are critically involved in the mechanism of sperm release.

Key words. Cytochalasin; apyrene sperm; eupyrene sperm; period gene; *Spodoptera littoralis*; RNAi.

Many life functions in organisms ranging from bacteria to humans display circadian (daily) rhythms that allow the temporal synchronization of metabolism, physiology and behavior. These rhythms are generated by a system of molecular feedback loops known as circadian clocks, or oscillators [1]. Clock genes involved in biotiming share substantial structural and functional homology in animals from insects to mammals [2]. The circadian system of animals consists of a network of oscillators; central, brain-located clocks regulate behavior and homeostatic functions, while peripheral oscillators located in different body organs appear to regulate a variety of organ-specific functions such as metabolic activity in mammalian liver [3] and olfactory responses in insects [4]. Recent studies have revealed that the expression of many genes is regulated in a circadian fashion [5]; however, rhythmic output pathways leading from the oscillators to overt rhythms are far from being understood [6, 7]. In particular, very little is known about cellular activities underlying physiological rhythms.

The peripheral circadian system involved in reproductive physiology has been described in several moth species. The release of sperm from the testes into the upper vas deferens (UVD) is restricted to a few hours within each 24-h light-dark (LD) cycle [8–11]. Temporal regulation of sperm migration is brain independent; the rhythm is self-sustained in constant conditions and can be entrained by light in isolated testis-UVD complexes in vitro [11, 12]. Consistent with local timing mechanisms, a canonical clock gene, *period* is rhythmically expressed in the moth testis-UVD complex [13]. Disruption of clock func-

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tion by constant light abolishes the rhythm of sperm release and related rhythms associated with sperm maturation in the UVD, leading to male sterility [14–17].

To study cellular mechanisms of sperm migration out of the testes, we used males of the cotton leafworm, *Spodoptera littoralis*, which display robust sperm release rhythms [11]. In this species, as in other moths, sperm cells differentiate and elongate in clonal bundles surrounded by an envelope of somatic cyst cells. Spermatogenesis results in two types of sperm bundles: eupyrene bundles, containing nucleated spermatozoa that correspond to fertilizing sperm of other animals, and apyrene bundles, containing spermatozoa which lose their nuclei due to meiotic chromosome missegragation [18]; the functions of the latter bundles are not understood [19]. Mature apyrene and eupyrene sperm bundles are released in daily batches; in the 16:8 LD cycle, the release of apyrene sperm bundles starts a few h before lights-off at Zeitgeber time (ZT) 8. The release of eupyrene sperm bundles starts 2 h later at ZT 10 and lasts for approximately 4 h [11]. Light and electron microscopy studies in *Spodoptera* and other moths have revealed that during the release process, sperm bundles penetrate a barrier of epithelial cells (barrier cells) that separates the testis from the UVD lumen [11, 20].

The cellular mechanisms underlying migration of large sperm bundles out of the testes at specific times in the LD cycle are not understood. In this paper, we studied the involvement of actin in the process of sperm release. Cytoplasmic β-actin is a major cytoskeletal component with dynamic properties dependent on the assembly of filamentous F-actin from G-actin monomers. Eukaryotic cells use actin polymerization to change shape, move and form contractile structures. Actin-based motility is linked to the rapid turnover of actin filaments, which is controlled by a large number of regulatory proteins [21, 22]. We used a fluorescent F-actin probe to examine the interactions between sperm bundles and barrier cells throughout the LD cycle. We then showed that inhibition of actin polymerization at a specific time of day prevents sperm release. Finally, we tested directly the relationship between actin and sperm release by introduction of double-stranded RNA (dsRNA) corresponding to a fragment of the *β-actin* gene. RNA-mediated interference (RNAi) is a novel tool used to study gene functions in a number of organisms, including those not suitable for traditional genetic studies [23]. RNAi causes specific degradation of target mRNA in affected cells and thus provides a powerful approach for understanding the genetic bases of different physiological processes. However, the delivery of dsRNA remains a problem in stages other than embryonic. Injection of dsRNA into adult stages has only been successful in *Caenorhabditis elegans* resulting in a transient interference with gene expression. In this study, injection of dsRNA corresponding to β-actin into the organ of adult moth allowed us to determine that actin polymerization in specific target cells is critically important for the process of sperm release.

Materials and methods

Insect breeding and organ preparation

Cotton leaf worms *S. littoralis*, were reared as described previously [15] under a 16:8 LD photoperiod at 25 °C. The dark phase started at ZT 12 and the light phase at ZT 20. The testis with the attached UVD (testis-UVD complex) was dissected in physiological saline [24] and cultured in vitro in Grace's medium (BioWhittaker) as previously described [11]. To quantify the number of released eupyrene sperm bundles, the wall of the UVD was cut open, eupyrene sperm bundles were dispersed and counted under a dissecting microscope. Apyrene spermatozoa dissociate from the bundles soon after entering the UVD; these bundles could not be quantified and were recorded as present or absent instead.

Histochemical studies of sperm release

Paraffin sections of testis-UVD complexes were prepared as described previously [15]. After the paraffin removal and rehydratation, sections were blocked for 1 h with 1% bovine serum albumin (BSA) in 0.1 M Na-phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (PBST). Blocking solution was then replaced with PBST containing 0.1% BSA and the F-actin-labeling fluorescent probe, Alexa Fluor Phalloidin (Molecular Probes) at a final concentration of 40 nM. Following overnight incubation at 4 °C, sections were washed three times in 0.03% PBST, twice in H_2O and mounted in Vectashield (Vector Laboratories) containing the nuclear marker DAPI. A similar procedure was used to examine the morphology of individual sperm bundles with the addition of 1 h incubation in 20 nM of the mitochondrial marker MitoTracker Green (Molecular Probes) in PBS before the final tissue wash in H_2O. Slides were examined under a DMBR Leica microscope, and images were acquired using a SPOT digital camera (Diagnostic Instruments). Selected preparations were viewed on a Leica TCS4D confocal microscope.

Cytochalasin treatments

Cytochalasin E (CE) from *Aspergillus clavatus* (Calbiochem) was dissolved in DMSO for a 10 mM stock solution. Testis-UVD complexes were dissected and incubated in Graces' medium containing 10^{-6}, 10^{-5} or 10^{-4} M CE for 4 h from ZT 8 to ZT 12 and then in fresh Grace's medium. At 4, 8 and 32 h after exposure to CE, released sperm bundles were counted in eight to ten preparations. To determine whether the effects of CE were time dependent, three groups of testis-UVD complexes were incu-

bated with 10^{-5} M CE at ZT 4–8, ZT 8–12 and ZT 12–16. Sperm bundles released into the UVD were counted every 4 h, during the first and second day in culture.

Synthesis of the riboprobe for β-actin

Based on the sequence of the *β-actin* gene from *S. littoralis* (AN Z46873), we amplified a fragment of this sequence by PCR using the following oligonucleotide primers 5′-GAGCAAGAGGTATC (sense primer) and 5′-AGTCCAGGACGATAC (antisense primer). A T3 promoter sequence was added to the 5′ end of the sense primer, and a T7 promoter was added to the 5′ end of the antisense primer. Template DNA was amplified by RT-PCR. The DNA fragment was gel-purified and used for in vitro transcription. This template was also used to prepare a radioactive probe for Northern blots.

Preparation of double-stranded actin RNA

To produce dsRNA corresponding to the *β-actin* gene, the DNA template described above was transcribed in vitro in two separate reactions with T3 and T7 RNA polymerases. After 1.5 h of transcription, the DNA template was destroyed with DNAse to stop the reaction. The efficiency of in vitro transcription was verified on agarose gels. Sense and antisense RNA strands were mixed in equal amounts, heated at 95 °C for 1 min, and slowly cooled at room temperature for 18 h. A small aliquot of annealed RNA was run on an agarose gel to confirm that most RNA has been annealed. Usually almost 100% of RNA used showed a shift in electrophoretic mobility, corresponding to dsRNA. Approximately 0.2 fmol of dsRNA was injected into each UVD. The testis-UVD complexes were injected with 1 µl of dsRNA or appropriate control via a pulled-glass micropipette attached to rubber tubing which allowed precise delivery of the required volume by gentle pressure. Each of the paired UVD was separately injected (near its junction with the testes) under a dissecting microscope in a sterile hood. Injected preparations were incubated in Grace's medium and sperm bundles released into the UVD were counted at designated times.

Quantitative PCR

Profiles of actin mRNA were measured by real-time RT-PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Two-step RT-PCR using the SYBR Green PCR Master Mix was performed with 2–3 µg of total RNA extracted from testes-UVD borders after injection of double-stranded actin RNA. PCR conditions and data analysis were based on the manufacturer's recommendations. 28S RNA was used as an internal standard. All data were normalized with this standard. Primers for quantitative PCR for actin were as follows: 5′-TGGGACGACATGGAGAAGATCT (sense) and 5′-GGGGGAGTTGAAGGTCTCAAA (antisense). Primers for 28S RNA were as follows: 5′-TCGGACGGTAGTTCTGACGAA (sense) and 5′-GCACACACGTCCGCACTATG (antisense).

Results

Cellular changes associated with sperm release rhythm

Testis follicles, which contain sperm bundles, are separated from the UVD by a barrier of epithelial cells (fig. 1A). The temporal pattern of sperm release was analyzed in longitudinal sections of testis-UVD complexes fixed at different times of the day and stained with a fluorescent F-actin probe, phalloidin, and the nuclear marker DAPI (fig. 1B–E). At ZT 4 (middle of the light phase), the UVD lumen was separated from the testis by a continuous layer of barrier cells with large and flattened cell nuclei (fig. 1B). Four hours later, at ZT 8, the continuity of this cellular barrier was disrupted by exiting apyrene sperm bundles. These bundles migrated in a folded state either singly or in groups (fig. 1C, arrows). Four hours later, at ZT 12, the UVD lumen contained apyrene bundles. At this time, a number of eupyrene sperm bundles, with DAPI-stained nuclei (fig.1D, arrowheads), were leaving the testes. The release of sperm bundles ceased by ZT 16; at this time, a continuous epithelial barrier again separated the testis from the UVD (fig.1E). Preparations fixed at ZT 20 and ZT 24 also displayed a uninterrupted barrier at the testis-UVD border (data not shown), consistent with the previous report showing that no sperm release occurs at these times [11].

To analyze the morphology of individual eupyrene and apyrene bundles in more detail, we stained them with phalloidin, Mitotracker and DAPI (fig. 1F–H). Pre-release, eupyrene bundles were enveloped in numerous cyst cells characterized by small nuclei (fig. 1F), including a head cyst cell situated above sperm tips. Spermatozoa were tightly aligned in bundles with thin elongated nuclei occupying the middle region. A strong actin-staining signal was observed in the nuclear region and in the area containing sperm tips. The latter may represent sites of attachment between spermatozoa and the cyst cell, similar to ectoplasmic specializations of mammals [25].

Apyrene bundles contained within the testis were also enveloped in several cyst cells and the head cyst cells at the tip of each bundle contained a distinct actin-rich protrusion (fig. 1G, H). Bundles located deep within the testes were fully elongated (fig. 1G), while those near the epithelial barrier were folded just below the area of actin concentration (fig. 1H). This is consistent with the observation that apyrene bundles leave the testis in the folded state (fig. 1C). To determine whether the levels of F-actin in sperm bundles fluctuate as a function of the time of day, we used phalloidin to stain bundles fixed at ZT 8, 16

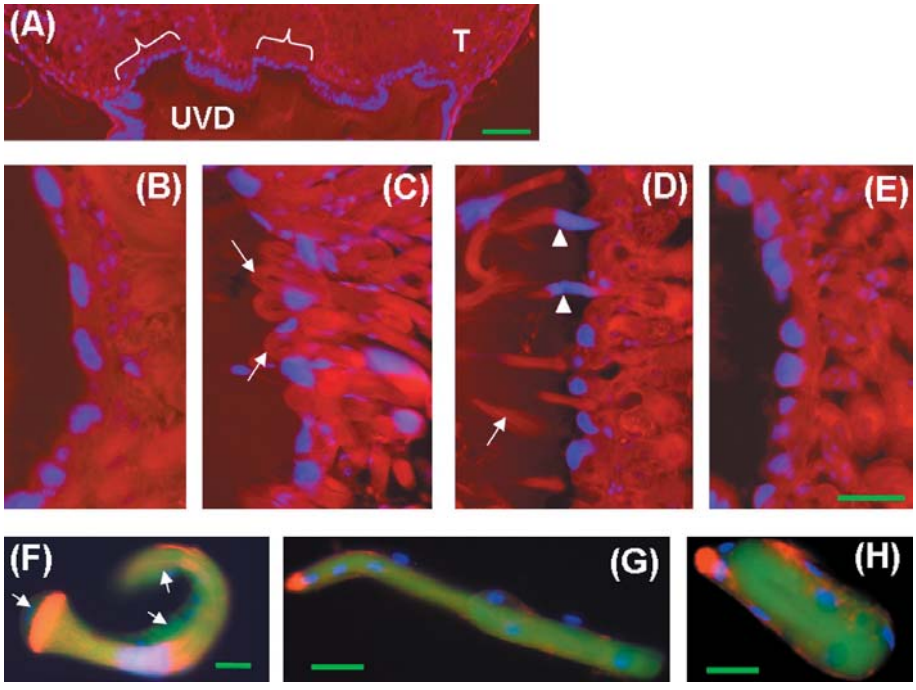

Figure 1. Cellular events associated with sperm release. (*A–E*) Sections stained with phalloidin-Alexa 594 and DAPI. (*A*) Border between the testis (T) and the UVD. Barrier cells separating lumen of individual testicular follicles from the UVD are marked with brackets. (*B–E*) Testis-UVD border in higher magnification at ZT 4 (*B*), ZT 8 (*C*), ZT 12 (*D*) and ZT 16 (*E*). Apyrene bundles are marked with arrows, eupyrene bundles with arrowheads. (*F–H*) Sperm bundles stained with phalloidin-Alexa 594, Mitotracker Green and DAPI. (*F*) Eupyrene sperm bundle before release; cyst cells are marked with arrows. (*G*) Unfolded apyrene sperm bundle. (*H*) Folded pre-release apyrene bundle. Bar, 100 µm in *A*; 20 µm in *B–H*.

and 24. We compared digital images (taken at the same exposure) of 50 eupyrene and 50 apyrene bundles derived from five testes per time point. No discernible changes in the intensity of the actin signal were observed between these time points (data not shown).

Effects of cytochalasin on sperm release

The observation that sperm bundles contain areas of concentrated F-actin suggests that actin polymerization may be important for sperm release. Consequently, we tested whether sperm release is affected by cytochalasin, a compound that inhibits the assembly of actin polymers. In the initial experiment, testis-UVD complexes were incubated for 4 h in Grace's medium containing different concentrations of CE. Incubation in 10^{-6} M CE had no effect on sperm release. The release was inhibited for 1 day by 10^{-5} M CE and for at least 2 days by 10^{-4} M CE (table 1). This demonstrated dose-dependent effects of CE on sperm release.

To determine whether the effects of CE were dependent on the time of its application, testis-UVD complexes were incubated with 10^{-5} M CE at three different times of the day. Sperm release occurred at the predicted time of day in the control preparations incubated in Grace's medium (fig. 2A). Incubation of testis-UVD with CE several h prior to the release gate (ZT 4–8) did not prevent sperm release (fig. 2B). In the organs that were exposed to CE during the release gate (ZT 8–12), sperm release was almost completely abolished but resumed on the second day within the appropriate circadian gate (fig. 2C). Treatment with CE at a later time (ZT 12–16) did not prevent sperm release (fig. 2D); however, there was a significant

Table 1. Effect of CE on sperm release.

Time	Average number (± SE) of sperm bundles in the UVD			
	solvent	10^{-6} M CE	10^{-5} M CE	10^{-4} M CE
Day 1, ZT16	55.5 (± 10.0)	49.0 (± 2.49)	0.3 (± 0.3)	0.1 (± 0.1)
Day 2, ZT16	46.7 (± 2.5)	38.1 (± 3.6)	40.7 (± 3.1)	2.0 (± 1.2)

Testis-UVD complexes were cultured in vitro and exposed to different concentrations of CE or solvent (DMSO ~ 10^{-2} M) for 4 h (ZT 8–12).

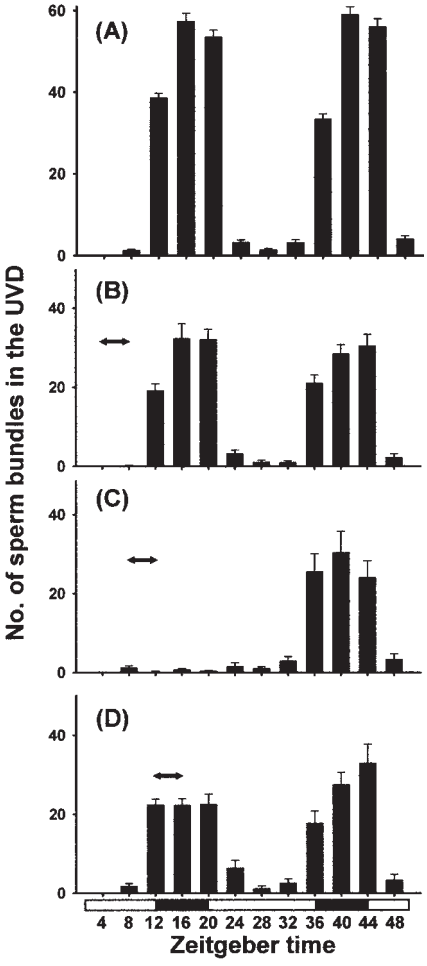

Figure 2. Effects of CE on sperm release. The testis-UVD complexes were cultured in vitro and treated with 10^{-5} M CE for 4 h at different times of the circadian cycle, as indicated by double arrows. Each bar represents the average number (± SE) of released eupyrene sperm bundles (n = 11–16). (*A*) Organs exposed to solvent (DMSO); data combined from control groups. (*B–D*) Organs exposed to CE at ZT 4–8 (*B*), ZT 8–12 (*C*) and ZT 12–16 (*D*).

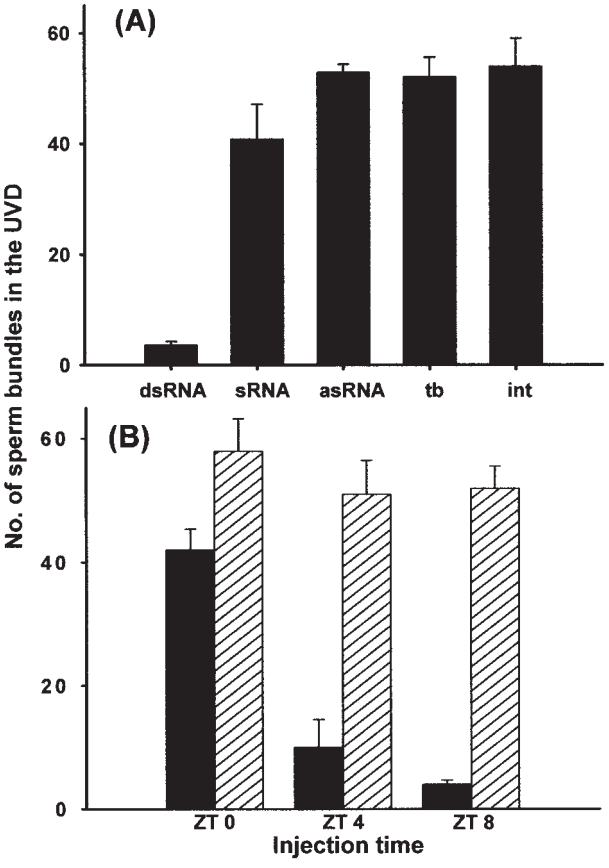

Figure 3. Effects of injection of double-stranded actin RNA on sperm release. Each bar represents the average number (± SE) of released eupyrene sperm bundles (n = 8–20). (*A*) Testis-UVD complexes were injected at ZT 8 with double-stranded actin RNA (dsRNA), sense RNA (sRNA), antisense RNA (asRNA), transcription buffer (tb) or were left intact in the culture (int). (*B*) Effects of double-stranded actin RNA (black) or tb (striped) injected at different ZT in the LD cycle. Sperm bundles were counted at ZT 16.

decline in the number of released sperm bundles compared to the controls. These data demonstrate that actin polymerization during a specific time of the day plays a critical role in the sperm release mechanism.

Effect of double-stranded β-actin RNA on sperm release

To more specifically probe the role of cytoplasmic actin in sperm release, we attempted to inhibit the expression of the *actin* gene through RNAi. Initially, we introduced β-actin dsRNA into the culture medium containing testis-UVD complexes but this treatment did not affect sperm release. We reasoned that the basement membrane and muscles surrounding the testis-UVD complex [26] may prevent entry of dsRNA to target cells. Therefore, we injected dsRNA into the UVD lumen where it would have more direct access into the cells at the testis-UVD border.

Injections were performed at ZT 8 and the released eupyrene sperm bundles were counted at ZT 16. Injection of dsRNA into the UVD inhibited the descent of apyrene sperm into the UVD and dramatically reduced the average number of released eupyrene sperm bundles (fig. 3 A). Control injections of corresponding sense RNA, antisense RNA and buffer did not produce statistically significant inhibition of sperm release compared to non-injected testis-UVD complexes cultured in parallel. We also tested whether the observed effects of actin dsRNA were specific, and not due to introduction of any dsRNA. Injections of dsRNA derived from a plant virus and dsRNA derived from a fragment of *S. littoralis* 3-dehydroecdysone 3β-reductase did not affect sperm release (data not shown).

We determined that the effect of actin dsRNA on sperm release was dependent on the time of injection (fig. 3 B). When dsRNA was injected at ZT 4, sperm release was also significantly reduced. However, injection at ZT 0, that is 8 h before the projected sperm release gate, did not

inhibit this process. The inhibition of sperm release was reversed on the second day after injection of dsRNA (data not shown), demonstrating that the effect was transient and the tissue remained viable. Given that the inhibition of sperm release by cytochalasin or by dsRNA depends on the time of day, we asked whether there are daily fluctuations in actin mRNA. We analyzed the steady-state levels of cytoplasmic actin RNA in the testis-UVD barriers collected every 4 h. Results of Northern blots and real-time RT-PCR did not reveal any significant changes in the levels of actin RNA throughout the 24-h LD cycle (data not shown).

Injection of dsRNA reduces the levels of β-actin mRNA

To determine whether injections of dsRNA suppressed the activity of the targeted gene, we measured the levels of β-actin mRNA by real-time RT-PCR in dsRNA-injected tissues. Total RNA for analysis was extracted from testis-UVD barriers dissected 0.5–3 h after injection of dsRNA or buffer. A substantial decline in the levels of actin mRNA occurred 0.5 h after injection (fig. 4); at this time, mRNA levels were reduced by approximately 45% compared to the control. One h after injection, the level of β-actin mRNA was still significantly lower in dsRNA-injected than in buffer-injected tissue. Partial recovery of actin mRNA levels was observed 2 h after injection, and almost full recovery occurred 3 h post-injection. Thus, we verified that dsRNA caused a prompt and significant decline in the levels of actin mRNA; this is consistent with the fact that the most effective inhibition of sperm release was achieved when dsRNA was injected just prior to the predicted release time (fig. 3).

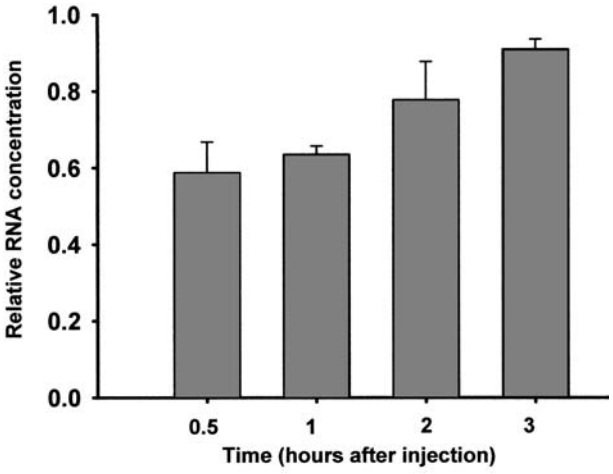

Figure 4. Injection of dsRNA into UVD causes a decrease in actin mRNA in testes barriers. dsRNA was injected at ZT 8. Tissues were collected after different times of incubation, as indicated. Total RNA was extracted and used for quantification in real-time RT-PCR. Bars represent average (± SE) RNA levels from three to five repetitions per time point.

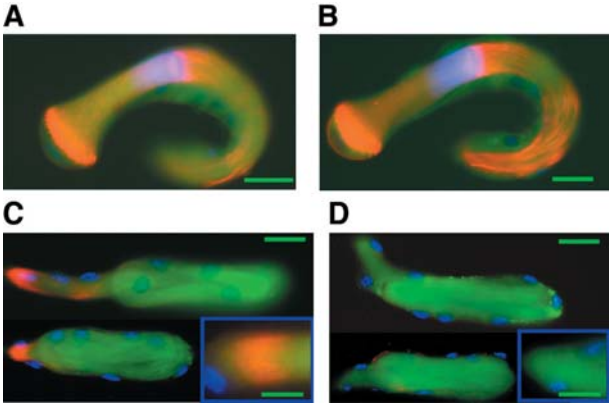

Figure 5. Injection of actin dsRNA caused site-specific depletion of F-actin from the head region of apyrene bundles. Preparations were injected with buffer or actin dsRNA at ZT 8 and 2 h later were fixed and treated with phalloidin-Alexa 594, Mitotracker Green and DAPI. The experimental and control group each consisted of five testis-UVD complexes; analysis of 20 apyrene and 20 eupyrene bundles from each testis (total of 400 bundles) showed comparable levels of F-actin within each group. Panels show representative eupyrene bundles injected with buffer (*A*) or dsRNA (*B*), and apyrene bundles injected with buffer (*C*) or dsRNA (*D*). Bars, 20 µm; bars in inserts, 5 µm.

Injection of dsRNA causes selective depletion of F-actin in the apyrene bundles

Disruption of sperm release by RNAi is correlated with the reduction in actin mRNA levels in the testis-UVD border region (fig. 4). The minimal preparation for RNA extraction contained eupyrene and apyrene sperm bundles, barrier cells and parts of the testis and the UVD wall (see fig. 1A). As β-actin may have different rates of turnover in these tissues, we needed to determine how different cells involved in sperm release were affected by RNAi at the level of F-actin. Testis-UVD complexes were injected with actin dsRNA or buffer at ZT 8; 2 h later, barrier regions were fixed and stained with phalloidin. There were no apparent differences in the organization and levels of F-actin in barrier cells from dsRNA- or buffer-injected preparations examined in the confocal microscope (data not shown). Likewise, F-actin in eupyrene bundles and unfolded apyrene bundles was not affected by RNAi; these bundles were indistinguishable from the control bundles (fig. 5A, B). In contrast, dsRNA injection resulted in dramatic depletion of F-actin in folded pre-release apyrene sperm bundles. In particular, the actin-rich cap was completely missing from head cyst cells following RNAi in all examined bundles while it was retained in all bundles from buffer-injected preparations (fig. 5C, D). This actin cap, then, is most likely involved in the sperm release process. The actin in the head region of the apyrene bundle may have low stability and a fast rate of turnover, such that lowering the pool of actin mRNA had an immediate effect on the levels of actin protein.

Discussion

Our study provided important insights into the cellular mechanism underlying rhythmic sperm release in moths. A daily rhythm of sperm release seems to involve clock-controlled interactions between sperm bundles and the cellular barrier at the testis base. This barrier prevents sperm release throughout most of the LD cycle except for a few hours before lights-off. During this circadian gate, apyrene sperm bundles penetrate the barrier followed by eupyrene sperm bundles. We revealed that apyrene bundles undergo folding prior to their migration through the cellular barrier and demonstrated conspicuous accumulation of actin filaments in the head cyst cell at the proximal end of each bundle. Several lines of evidence suggest that the actin-rich cap may play a critical role in sperm release.

Apyrene bundles, which contain anucleated spermatozoa, have been reported in all Lepidoptera; however, their functions are uncertain [27]. Based on the RNAi experiments, we suggest that these bundles are instrumental for the initiation of sperm release. Apyrene sperm bundles carry a protruding actin cone in the head cyst cell, which envelopes tips of spermatozoa. Injection of double-stranded actin RNA, which resulted in complete depletion of F-actin from folded apyrene bundles, inhibited the release of both apyrene and eupyrene sperm. A selective decline of F-actin in the head cyst cell suggests that this actin may be involved in cellular motility, which requires a rapid turnover of actin filaments [28, 29]. Elongation of actin filaments is driven by the high concentration of actin monomers [30]. We hypothesize that sperm release depends on rapid actin polymerization that cannot be achieved by subunit recycling but requires recruitment of monomers via de novo actin translation. Reduction of actin mRNA levels via RNAi may have led to a shortage of new G-actin subunits at the specific site of movement-related actin polymerization. This hypothesis is consistent with reports that motility in fibroblasts is augmented by the translation of β-actin from mRNA localized just proximal to the cell leading edge [31]. RNAi apparently revealed the site of rapid actin translation and polymerization in apyrene bundles that is critically important for sperm release. A similar depletion of actin was not detected in eupyrene bundles. We speculate that actively migrating apyrene bundles may leave the exit channels in the testis-UVD barrier, which are then used by eupyrene bundles. This scenario is consistent with our current data and previous report [11] that apyrene bundles always precede eupyrene bundles en route from the testis into the UVD.

The output pathway leading from the circadian oscillator to the overt rhythm of sperm release is not understood. Time-dependent effects of cytochalasin and actin RNAi on sperm migration suggest that some factors in the circadian output pathway may be involved in regulating actin dynamics in apyrene bundles. Regulation of actin treadmilling could be controlled in a number of ways. We have found that steady-state levels of actin mRNA do not cycle in the testes-UVD border; likewise, the levels of F-actin in apyrene bundles do not appear rhythmic. However, several factors, including ATP, Ca^{2+} and many actin-binding proteins, such as the Arp2/3 complex and multiple nucleation-promoting factors [21, 28], could be regulated by the circadian clock to facilitate temporally coordinated sperm release. The circadian clock could also influence specific signal transduction cascades regulating adhesive properties of sperm bundles and barrier cells. Consistent with these speculations, the clock gene *period* (*per*) is expressed in the cyst cells enveloping sperm bundles and in the barrier cells [13; P. Bebas, unpublished data]. Recently, we sequenced a fragment of the *per* gene from *S. littoralis* (GenBank accession number AF315349) and injected testis-UVD complexes with dsRNA corresponding to a *per* mRNA fragment. Results of this RNAi experiment suggest that interference with *per* expression inhibits sperm release [B. Gvakharia and P. Bebas, unpublished data]. This technique may allow us in the future to identify clock-controlled genes that interact with actin in the process of sperm release.

Our data indirectly suggest rhythmic regulation of actin dynamics in specific animal cells. Clock-controlled cytoskeletal changes have been reported in plants; namely, the rhythmic transcription of tubulin is involved in the circadian rhythms in plants and animals [32–34]. Interestingly, recent microarray analyses of clock-controlled genes revealed the rhythmic expression of several genes encoding actin-associated proteins in *Drosophila* [35] and mouse [36]. This suggests that circadian control of actin dynamics could be involved in a variety of cellular rhythms.

The involvement of actin in sperm release may be evolutionarily conserved. The prevention of actin polymerization has been reported to interfere with sperm release from seminiferous tubules in rats [37]. Actin-rich structures called ectoplasmic specializations serve as attachments between maturing spermatozoa and Sertoli cells [25] and turnover of these junctions is involved in mammalian sperm release [38]. Cyst cells enveloping insect spermatozoa are considered homologous with Sertoli cells [39]. Our current study suggests that there may be some degree of functional homology in sperm release mechanisms between insects and mammals, at least with regard to the involvement of actin.

In summary, we present several lines of evidence suggesting that actin polymerization underlies the rhythmic release of sperm from moth testes. Combined physiological and molecular results of RNAi experiments lead to the hypothesis that actin-rich structures in the head cyst cells of apyrene bundles may provide a driving force for migration of sperm bundles. The mechanism of this migration will be the subject of future studies.

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