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Effects of *period* RNAi on V-ATPase expression and rhythmic pH changes in the vas deferens of *Spodoptera littoralis* (Lepidoptera: Noctuidae)

Joanna Kotwica-Rolinska [a], Barbara O. Gvakharia [b], Urszula Kedzierska [a], Jadwiga M. Giebultowicz [c], Piotr Bebas [a,*]

[a] *Departament of Animal Physiology, Zoological Institute, Faculty of Biology, University of Warsaw, 1 Miecznikowa Str., 02-096 Warsaw, Poland*
[b] *Department of Botany and Plant Pathology, Oregon State University, 2082 Cordley Hall, Corvallis, OR 97331-2902, USA*
[c] *Department of Zoology, Oregon State University, 3029 Cordley Hall, Corvallis, OR 97331-2914, USA*

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ABSTRACT

Circadian clocks (oscillators) regulate multiple aspects of insect behaviour and physiology. The circadian system located in the male reproductive tract of Lepidoptera orchestrates rhythmic sperm release from testis and sperm maturation in the upper vas deferens (UVD). Our previous research on the cotton leafworm, *Spodoptera littoralis*, suggested rhythmic changes in the V-ATPase levels in the UVD epithelium, which correlated with rhythmic pH fluctuations in the UVD lumen. However, it was not known whether UVD cells contain clock mechanism that generates these daily fluctuations. In the current paper, we show circadian rhythm in the expression of clock gene *period* at the mRNA and protein level in the UVD epithelium. To determine the role of PER in V-ATPase and pH regulation, testes–UVD complexes were treated *in vitro* with double-stranded fragments of *per* mRNA (dsRNA). This treatment, which transiently lowered *per* mRNA and protein in the UVD, altered expression of V-ATPase c subunit. In addition, *per* RNAi caused a significant delay in the UVD lumen acidification. These data demonstrate that the UVD molecular oscillator involving the *period* gene plays an essential role in the regulation of rhythmic V-ATPase activity and periodic acidification of the UVD lumen.

1. Introduction

Numerous biological processes display daily rhythmic fluctuations that are regulated by circadian clocks. Circadian rhythms are entrained by environmental signals, mainly light/dark and temperature cycles (known as Zeitgebers), but also free-run in the absence of such signals with a circa 24 h period. The clock mechanism is cell-autonomous and involves rhythmic expression of clock genes sharing structural and functional homologies from insects to mammals. The expression of clock genes and proteins occurs in a feedback-loop, which is best understood in the fruit fly *Drosophila melanogaster*. At the core of the *Drosophila* circadian clock there are four clock genes *Clock* (*Clk*), *cycle* (*cyc*), *timeless* (*tim*), and *period* (*per*) (Hardin, 2011; Sandrelli et al., 2008). The expression levels of *per* and *tim* are regulated by transcriptional activators encoded by *Clk* and *cyc*. This leads to periodic increase in the levels of *per* and *tim* mRNA and PER and TIM proteins. The latter accumulate in cell nuclei, and repress CLK/CYC activators, leading to suppression of *per* and *tim* transcription. Subsequent degradation of TIM and PER proteins leads to de-repression of CLK/CYC and onset of the next molecular oscillator cycle. The molecular clock mechanism has been investigated in several other insect species, revealing considerable differences from the *Drosophila* model, for example, one of two *cryptochrome* genes (*Cry2*), appears to work as a transcriptional repressor replacing the *tim* gene in this role in the honey bee and Monarch butterfly (Tomioka and Matsumoto, 2009). Among clock genes, *period* (*per*) appears to play a conserved role as it displays cycles in mRNA and protein expression in all insects investigated so far (Tomioka et al., 2012). However, there are differences concerning subcellular distribution of PER in clock containing cells. Immunohistochemical analysis showed that in the brain neurons of the firebrat, mayflies, damselflies, stoneflies, caddisflies, crickets, cockroaches and moths, PER protein is predominantly cytoplasmic (Sauman and Reppert, 1996; Sehadova

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* Corresponding author. Tel.: +48 22 554 1026; fax: +48 22 554 1030.
E-mail addresses: askako@biol.uw.edu.pl (J. Kotwica-Rolinska), gvakharv@science.oregonstate.edu (B.O. Gvakharia), u.kedzierska@biol.uw.edu.pl (U. Kedzierska), giebultj@science.oregonstate.edu (J.M. Giebultowicz), piotrbe@biol.uw.edu.pl (P. Bebas).

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et al., 2004; Shao et al., 2006; Wen and Lee, 2008; Yang et al., 2009; Zavodska et al., 2003). In contrast to the central nervous system, PER protein is located in the nuclei of peripheral moth tissues such as epithelium in the *Antheraea pernyi* midgut (Sauman and Reppert, 1998), olfactory neurons and epithelial cells in the antenna of *Manduca sexta* (Schuckel et al., 2007). In addition, strong 24 h rhythm of nuclear PER has been observed in the epithelial cells of male reproductive system in the Egyptian cotton leaf worm *Spodoptera littoralis* and the codling moth *Cydia pomonella* (Gvakharia et al., 2000; Kotwica et al., 2009a).

The biological clock in insects controls various behavioural processes, such as hatching from eggs, larval ecdysis, adult eclosion, rest/activity rhythms, and sensitivity of olfactory and taste receptors. Furthermore, circulating levels of many insect hormones are influenced by the circadian system (Bloch et al., 2012). The molecular and cellular basis of known *Drosophila* rhythms are well understood owing to available mutations in clock genes and techniques to manipulate clock function in a tissue specific fashion (Allada and Chung, 2010; Hardin, 2011). In other insect species, the RNAi method has been adapted recently to analyse involvement of clock genes in different rhythms (Terenius et al., 2011). Reduced *per* mRNA in the *Bombyx mori* transformed with a construct encoding *per* double-stranded RNA (*per* dsRNA) affected the egg hatching rhythm, and shortened time of development (Sandrelli et al., 2007). In the cricket, *Gryllus bimaculatus*, *per* dsRNA injection disrupted both the locomotor activity rhythm and the rhythm of electrical activity in the optic lobes in adults (Moriyama et al., 2008). Most recently *per* RNAi disrupted circadian behaviour was described in the adult males of the mangrove cricket, *Apteronemobius asahinai* (Takekata et al., 2012). Similarly, disturbance of locomotor activity rhythms has been observed in insects injected with dsRNA of *tim*, *cyc* and *clk* and the *pigment-dispersing factor* (*pdf*) genes (Hassaneen et al., 2011; Kamae and Tomioka, 2012; Lee et al., 2009). In the bean bug, *Riptortus pedestris* reduced expression of clock genes disrupted the cuticle deposition rhythm and significantly altered the incidence of reproductive diapause (Ikeno et al., 2011a). There is also evidence based on RNAi that circadian clocks regulate reproductive functions in the desert locust, *Schistocerca gregaria*. Locusts treated with *per* and *tim* dsRNAs showed significantly reduced fertility (Tobback et al., 2011, 2012).

The peripheral brain-independent circadian system localized in male reproductive system of lepidopteran species is involved in generating multiple circadian rhythms, that provide a temporal framework for coordinated release and maturation of sperm. Indeed, when circadian rhythms are suppressed by constant light, the moths become sterile due to diminished release of sperm (Bebas and Cymborowski, 1999; Giebultowicz et al., 1993, 1990; Riemann and Ruud, 1974). In moths with intact clock system, sperm bundles are released from the testis into the upper vas deferens (UVD) during the evening and early night. Sperm is held in the UVD lumen during the night and then transferred from the UVD to the seminal vesicles in the early morning. Such two-step rhythm of sperm release was first identified in the moth *Anagasta kuehniella* (Riemann et al., 1974) and later confirmed in males of the gypsy moth *Lymantria dispar* (Giebultowicz et al., 1988) the codling moth *C. pomonella* (Giebultowicz and Brooks, 1998) and the Egyptian cotton leaf worm *S. littoralis*(Bebas et al., 2001). These rhythms are self-sustained in constant darkness and can be entrained by light in isolated testis–UVD complexes *in vitro* suggesting the presence of peripheral oscillatory system in the male reproductive tissues (Bebas et al., 2001; Giebultowicz et al., 1989). The core clock gene *per* is rhythmically expressed in the testis of *C. pomonella* (Gvakharia et al., 2000), *B. mori* (Iwai et al., 2006) and *S. littoralis* (Kotwica et al., 2009a). In the latter species, *per* cycling is observed in the barrier cells separating the testis follicles from the UVD, and this gene was functionally linked to the rhythm of sperm release, as *per* dsRNA treatment inhibited sperm release from the testis (Kotwica et al., 2009a).

The peak of sperm accumulation in the UVD lumen of *S. littoralis* coincides with maximum secretion of glycoproteins (Bebas et al., 2002b) and with periodic acidification of this compartment (Bebas et al., 2002a). In our previous studies, we showed that spermatozoa released into the UVD lumen stay in contact with the seminal fluid, which is created by the secretory activity of the UVD epithelial cells (Bebas et al., 2002b). Glycoprotein composition in the seminal fluid changes in a rhythmic manner, suggesting rhythmic oscillations of the UVD epithelium activity. Furthermore, pH in the UVD lumen oscillates in a daily rhythms and this is associated with rhythmic fluctuations in the levels of the vacuolar type H^+-ATPase known as V-ATPase (Bebas et al., 2002a). V-ATPases are ubiquitous and highly conserved proton pumps present in eukaryotic cells, which energize membranes of specific organelles such as endosomes, lysosomes and secretory vesicles. V-ATPases found in the apical plasma membranes of different cells play the key role in pH homeostasis by creating luminal microenvironment in variety of tubular organs, such as kidney and epididymis (Brown and Breton, 2000). In insects, V-ATPases drive acidification/alkalisation of many extracellular compartments in digestive system such as midgut and salivary glands, labial glands, in Malpighian tubules and sensory sensilla (Wieczorek et al., 2009, 2000).

V-ATPases are multi-subunit complexes consisting of cytoplasmic V_1 domain that possess catalytic activity and a membrane-associated proton-conducting V_0 domain (Wieczorek et al., 2009). We have previously shown rhythmic changes in the protein level of the B subunit of V-ATPase V_1 domain in the upper vas deferens (UVD) epithelium of the moth *S. littoralis* (Bebas et al., 2002a). High levels of B subunit in the apical border of the UVD cells were associated with the acidification of the UVD lumen, which coincided with retention of spermatozoa in this compartment. Rhythmical changes in the V-ATPase B protein level, as well as acidification of seminal fluid in the UVD lumen were maintained in constant darkness, suggesting that they are under circadian control. In the current study, we investigated whether clock mechanism is localized in the UVD epithelial cells, and if so, whether this clock regulates expression and activity of V-ATPase subunits at the mRNA and protein levels. To address these questions, we analysed *per* expression in the UVDs of males kept under different light conditions. Our data suggest that molecular oscillator is localized in the UVD epithelial cells and it continues to function in the isolated reproductive system. Using *per* RNAi we showed differential effects of clock disruption on the expression of genes encoding B and c subunits of V-ATPase. Our study suggests that *per* based molecular clock in the UVD regulates rhythmic changes in the pH of seminal fluid.

2. Materials and methods

2.1. Insects rearing

Experiments were performed on 2-day-old males of the Egyptian cotton leaf worm, *S. littoralis*. Insects were reared as described previously (Bebas and Cymborowski, 1999), in cycles of 16 h light:8 h dark (LD) at 25 °C. Start of the dark period for this nocturnal species was set as Zeitgeber time 12 (Zt12) and start of the light phase at Zt20. In specific experiments, males were transferred into constant light (LL), or constant darkness (DD) one day prior to adult eclosion. Dissections of the reproductive system were carried out in physiological saline formulated for moths (Weevers, 1966). For *in vivo* experiment (detection of *per* transcript