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Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar H+-ATPase

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Summary

Recent studies have demonstrated that the peripheral tissues of vertebrates and invertebrates contain circadian clocks; however, little is known about their functions and the rhythmic outputs that they generate. To understand clock-controlled rhythms at the cellular level, we investigated a circadian clock located in the reproductive system of a male moth (the cotton leaf worm *Spodoptera littoralis*) that is essential for the production of fertile spermatozoa. Previous work has demonstrated that spermatozoa are released from the testes in a daily rhythm and are periodically stored in the upper vas deferens (UVD). In this paper, we demonstrate a circadian rhythm in pH in the lumen of the UVD, with acidification occurring during accumulation of spermatozoa in the lumen. The daily rhythm in pH correlates with a rhythmic increase in the expression of a proton pump, the vacuolar H+-ATPase (V-ATPase), in the apical portion of the UVD epithelium. Rhythms in pH and V-ATPase persist in light/dark cycles and constant darkness, but are abolished in constant light, a condition that disrupts clock function and renders spermatozoa infertile. Treatment with colchicine impairs the migration of V-ATPase-positive vesicles to the apical cell membrane and abates the acidification of the UVD lumen. Bafilomycin, a selective inhibitor of V-ATPase activity, also prevents the decline in luminal pH. We conclude that the circadian clock generates a rhythm of luminal acidification by regulating the levels and subcellular distribution of V-ATPase in the UVD epithelium. Our data provide the first evidence for circadian control of V-ATPase, the fundamental enzyme that provides the driving force for numerous secondary transport processes. They also demonstrate how circadian rhythms displayed by individual cells contribute to the synchrony of physiological processes at the organ level.

Key words: moth, vas deferens, pH, circadian clock, bafilomycin, cotton leaf worm, *Spodoptera littoralis*.

Introduction

Daily (circadian) rhythms observed at the molecular and cellular levels provide temporal organization of life functions and adapt organisms to a 24-h solar day (Pittendrigh, 1960; Dunlap, 1999). Daily rhythms are generated by the endogenous circadian clock, which is entrained by environmental signals, mainly light/dark cycles, and free-runs in the absence of such signals. This timing mechanism is cell-autonomous and involves rhythmic expression of several clock genes sharing structural and functional homologies among animals, from insects to mammals (Reppert and Weaver, 2000; Ripperger and Schibler, 2001). Clock genes are rhythmically expressed in the brain centers controlling behavioral rhythms and also in many peripheral tissues throughout the body. It has been demonstrated that the organs of *Drosophila melanogaster*, zebrafish and rat can maintain self-sustained cycling of clock genes *in vitro* (Plautz et al., 1997; Whitmore et al., 1998; Giebultowicz et al., 2000; Yamazaki et al., 2000). Moreover, even cultured fibroblasts display rhythmic expression of clock genes and clock-controlled transcription factors (Balsalobre et al., 1998; Yagita et al., 2001). These data suggest that local circadian clocks reside in the peripheral tissues of complex animals; however, their rhythmic outputs are poorly understood (Brown and Shibler, 1999; Giebultowicz, 2000). Especially lacking is an understanding of rhythmic cellular functions that may lead to physiological rhythms at the organ level.

A remarkable example of an autonomous peripheral clock is the circadian system located in insect male reproductive tissues. In several species of moth, the release of sperm from the testes occurs in a daily rhythm (Riemann et al., 1974; Giebultowicz et al., 1988; Bebas et al., 2001). Clones of differentiated spermatozoa (sperm bundles) are released from the testes into the upper vas deferens (UVD) in the evening, remain in the UVD lumen overnight and are subsequently delivered to the seminal vesicles in the morning, as shown schematically in Fig. 1. Rhythmic patterns of sperm movement persist, and can be entrained by light, in isolated testis/vas deferens complexes *in vitro* (Giebultowicz et al., 1989; Bebas

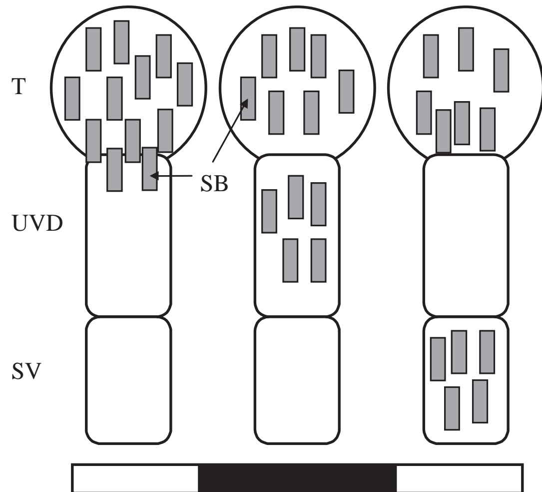

Fig. 1. Schematic representation of the rhythm of sperm release in moths. Sperm bundles (SB) descend from the testis (T) into the upper vas deferens (UVD) during a period of a few hours before and after lights-off. Sperm bundles are retained in the UVD lumen during the night and then transferred to seminal vesicles (SV) within a few hours after lights-on. During each daily cycle, the sperm bundles closest to the base of the testis are released in this stepwise fashion. Black and white bars represent, respectively, the dark and light phases of the 16 h:8 h L:D cycle.

et al., 2001), indicating that these organs contain a photoreceptive circadian system. This conclusion is supported by the observation that the essential 'gears' of the circadian clock, the gene *period* (*per*) and its protein PER (Dunlap, 1999), are rhythmically expressed in the cells forming the base of the testis and the epithelial wall of the UVD (Gvakharia et al., 2000).

The importance of the functional circadian clock for reproduction is manifest dramatically in male moths kept under conditions of constant light (LL), a condition that stops insect clocks (Saunders, 1982). The rhythms associated with sperm release are disrupted in LL conditions (Giebultowicz et al., 1988; Bebas et al., 2001), leading to a reduction in the quantity and quality of sperm produced (Riemann and Ruud, 1974; Giebultowicz et al., 1990; Bebas and Cymborowski, 1999).

We used the robust and accessible circadian system in the male reproductive tissues of a moth to investigate output rhythms generated by peripheral clocks. In particular, we sought to identify cellular rhythms in the UVD epithelium that might be associated with periodic retention of sperm in the UVD lumen. Data obtained from mammals indicate that the sperm ducts maintain a low luminal pH, which may be required for sperm maturation (Hinton and Palladino, 1995). We tested the pH of sperm ducts at different times of day and report here a circadian rhythm of acidification in the UVD lumen of the moth *Spodoptera littoralis*. We further reveal that the proton pump, the vacuolar H^+-ATPase (V-ATPase) (Nelson and Harvey, 1999), is rhythmically expressed in the UVD epithelium, with levels reaching a peak prior to the decline in luminal pH. Inhibition of V-ATPase activity, or interference with its accumulation in the apical region of the UVD epithelium, prevents luminal acidification of the UVD. We conclude that the circadian clock generates a rhythm in pH by regulating the levels and subcellular distribution of V-ATPase in the epithelium of the UVD.

Materials and methods

Insect rearing and measurement of pH

All experiments were performed on adult males of the cotton leaf worm *Spodoptera littoralis*. Insects were reared as described previously (Bebas and Cymborowski, 1999) in cycles of 16 h:8 h light:dark (LD) at 25 °C. The dark phase started at Zeitgeber time 12 (Zt12) and the light phase at Zt20. Dissections of the reproductive system were carried out in physiological saline formulated for moths (Weevers, 1966).

To measure luminal pH, UVDs from individual moths were opened in a small glass well, the luminal fluid (approximately 2 µl) was withdrawn and pipetted onto a pH microelectrode (PHR-146 micro combination pH electrode; Lazar Research Labs) and the pH was read using a standard pH meter. For each time point, pH values were measured from 10–12 moths and averaged. Before each series of pH data collection, 2 µl of reference buffers (pH 4.0 and 7.0) were pipetted onto the microelectrode to verify the accuracy of the system.

Protein detection

For western blots, equal numbers of UVDs per time point were homogenized in lysis buffer and centrifuged for 10 min at 12 000 ***g***. The protein concentration in the supernatant was measured using the Bio-Rad protein assay (Bio-Rad Laboratories), and equal amounts of protein were loaded onto polyacrylamide gels. Polypeptides resolved by electrophoresis were electroblotted onto PDV membrane (Millipore). Blots were incubated with a primary polyclonal antibody against the B-subunit of mosquito V-ATPase (Filippova et al., 1998), which labeled a protein of the expected size, 55–57 kDa. This protein was not labeled in blots incubated with the antigen-preabsorbed antibody, confirming its identity as the B-subunit of V-ATPase. Blots were incubated for 1 h in primary antibody diluted 1:1000 in blocking buffer containing 1 % bovine serum albumin (BSA) in 0.05 mol l^{-1} sodium-phosphate-buffered saline (PBS), washed twice in PBS and incubated for 30 min in a 1:1000 dilution of alkaline-phosphatase-conjugated anti-rabbit secondary antibody (Southern Biotechnology Inc). Blots were then washed twice in PBS, and the color reaction was developed according to the manufacturer's instructions (Roche). Pre-stained protein standards (Bio-Rad Laboratories) were used for estimates of the molecular masses of the blotted proteins.

To study the spatial distribution of V-ATPase, UVDs of *S. littoralis* were fixed in 4 % paraformaldehyde in 0.1 mol l^{-1} PBS at 4 °C for 2 h, dehydrated through an increasing ethanol series, embedded in paraffin and then cut into sections 6 µm thick. Following the removal of the paraffin (with toluene) and rehydration, sections were blocked for 1 h with 5 % normal

goat serum (NGS) and 0.1% BSA in 0.1 mol l^{-1} PBS containing 0.3% Triton X-100 (PBST). Sections were then incubated for at least 12 h at 4°C with polyclonal antibody generated against the B-subunit of V-ATPase diluted 1:6000 in DAKO antibody dilutent (DAKO Corp., USA), and washed three times in PBST. The DAKO EnVision System (DAKO Corp., USA) with anti-rabbit secondary antibody conjugated to alkaline phosphatase was used according to the manufacturer's instruction. Following counter-staining of cell nuclei with Mayer's hematoxylin, sections were washed in PBS and mounted in an aqueous mounting medium (DAKO Faramount). In some experiments, V-ATPase was detected using anti-rabbit antibody conjugated to Texas Red (Molecular Probes Inc., USA) diluted 1:1000 with PBST, containing 0.3% NGS and 0.1% BSA for 2 h at room temperature (22–24°C). Tissues were then washed three times in PBST and mounted in Vectashield medium containing DAPI (Vector Laboratories Inc., USA). As a negative control, primary antibody was preabsorbed with the antigen or omitted. Slides were examined under a DMBR Leica microscope, and images were captured using a SPOT digital camera (Diagnostic Instruments).

Injections

To determine the effects of bafilomycin on luminal pH, the UVDs of CO_2-anesthetised moths were injected at Zt8 with 1 µl of 200 nmol l^{-1} bafilomycin A_1 (Sigma), diluted in moth saline from DMSO-dissolved stock (final DMSO concentration 6%). Control UVDs were injected with 1 µl of 6% DMSO in saline. Moths were left in LD conditions until Zt16, at which time the pH was measured as described above.

To determine the effects of the microtubule-disrupting drug colchicine, males were injected in the abdomen with 1 µl of 0.9 mmol l^{-1} colchicine (Sigma) diluted in moth saline at Zt8. UVDs from colchicine- or saline-injected insects were dissected at Zt12 and Zt16. UVDs were used to measure luminal pH, as described above, and to examine the spatial distribution of V-ATPase. In the latter experiment, UVDs were fixed, sectioned and incubated with antibody against the B-subunit of V-ATPase, followed by Texas-Red-conjugated secondary antibody (Molecular Probes), as described above.

Results

Circadian rhythm of luminal pH in the vas deferens

We measured daily patterns of pH in the lumen of the vas deferens (UVD) of *S. littoralis* reared in light/dark cycles (LD), constant darkness (DD) and constant light (LL). In LD conditions, luminal pH fluctuated with a daily rhythm (Fig. 2A). Significant acidification of the UVD lumen occurred during the night, coinciding with periodic storage of sperm in this segment of the reproductive tract (see Fig. 1). The pH rhythm could be entrained by shifted LD cycles: a delay of 8 h in the LD schedule caused a similar delay in the decline in pH during the second day in the changed LD conditions (data not shown). A strong circadian pH rhythm with a difference of approximately 0.6 pH units between the peak and the trough persisted in moths that were kept in DD conditions for 2 days prior to dissection (Fig. 2B). However, the rhythm of UVD acidification was abolished in moths kept for 2 days in LL conditions (Fig. 2C).

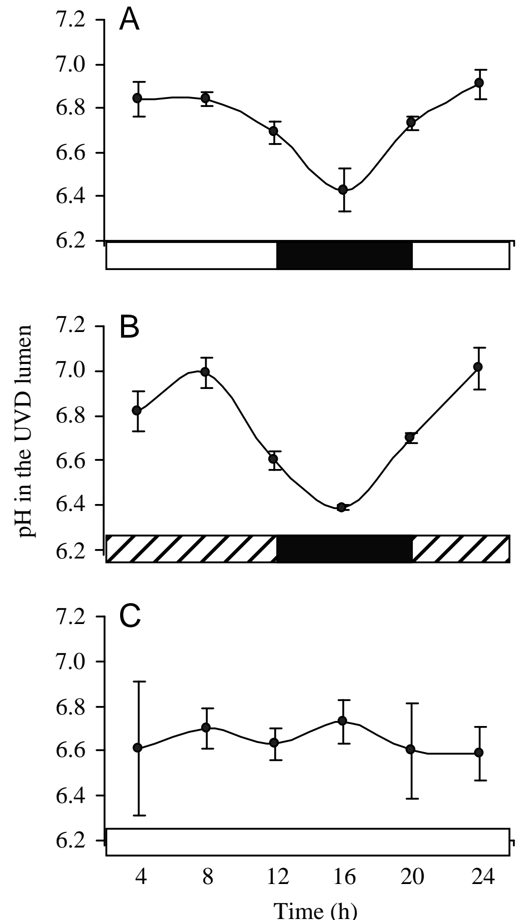

Fig. 2. Pattern of pH changes in the upper vas deferens (UVD) lumen of *Spodoptera littoralis.* (A) Under a 16 h:8 h L:D cycle, rhythmic changes are detected in the luminal pH, with significant acidification of the UVD occurring in the middle of the dark phase. (B) The robust pH rhythm continues on the second day in constant darkness. (C) The rhythm is abolished in constant light. Each data point represents the pH measured in 10 individuals. Values are means ± S.E.M. The horizontal bars represent the day (open), night (filled) and former day (hatched) portions of the photo-regime.

V-ATPase displays a circadian rhythm in quantity and subcellular distribution in the UVD epithelium

The principal enzyme regulating acidification in living systems is the multi-subunit proton pump V-ATPase (Nelson and Harvey, 1999), which is active in mammalian sperm ducts (Brown et al., 1997). To examine whether V-ATPase is expressed in the moth UVD, we performed western blots using a polyclonal antibody against the B-subunit of V-ATPase (Filippova et al., 1998). A 55–57 kDa protein corresponding to the B-subunit was detected in the UVD homogenates and showed daily fluctuations in abundance (Fig. 3A). Blots incubated with the antigen-preabsorbed antibody did not show a protein band of corresponding size, confirming the specificity

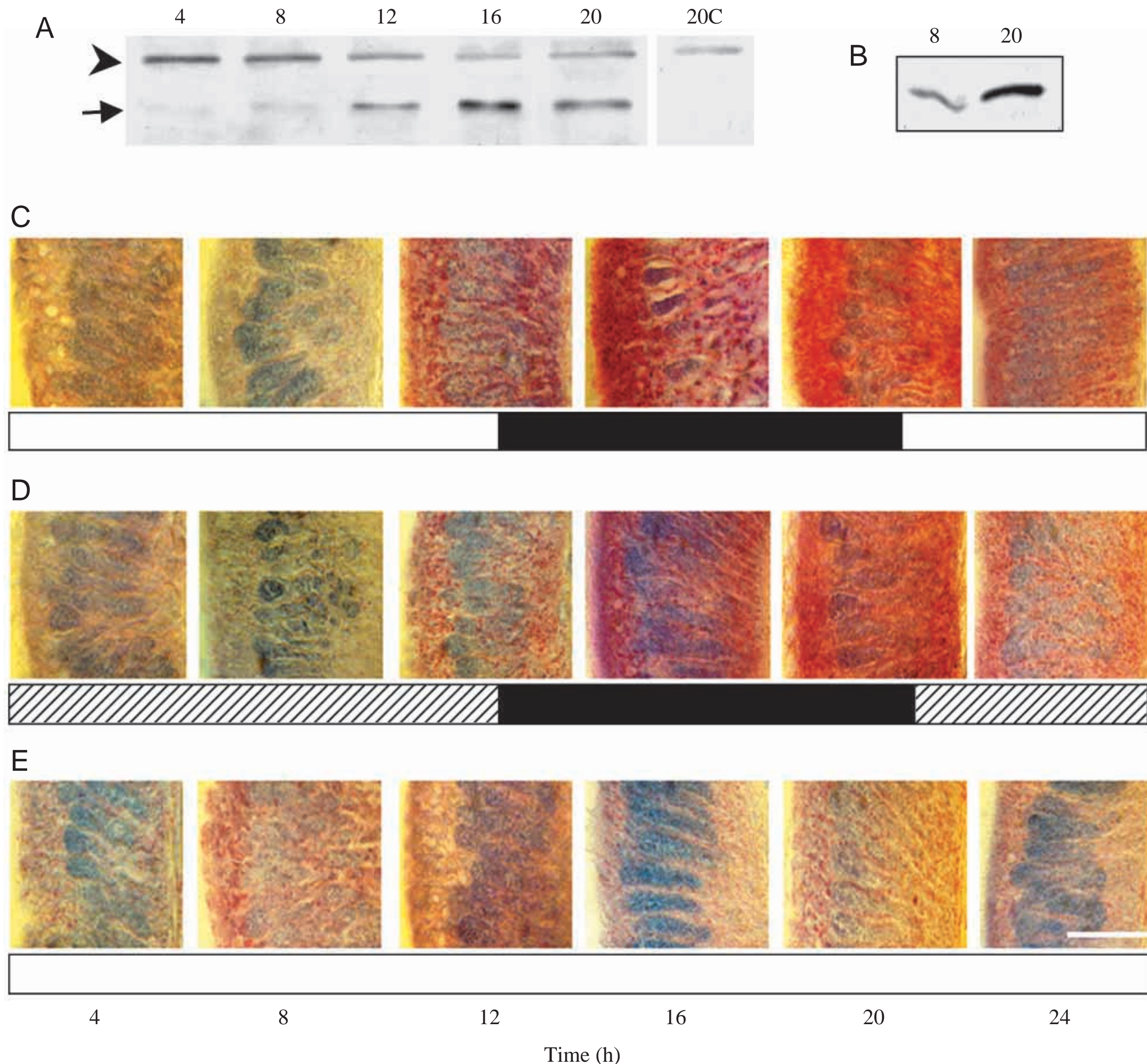

Fig. 3. Expression of the B-subunit of V-ATPase in the vas deferens of *Spodoptera littoralis.* (A) Western blot analysis of B-subunit levels (arrow) in the upper vas deferens (UVD) during a 16 h:8 h L:D cycle (LD) at the Zeitgeber times indicated above the blot. The protein levels (arrow) show daily oscillations. The lane marked 20C was treated with antigen-preabsorbed antibody. Non-specific protein (arrowhead) served as a loading control. (B) UVDs from moths kept in constant darkness (DD) maintained daily oscillations in B-subunit levels, with a lower level during former daytime at circadian time 8 (Ct8) and a higher level during former nighttime at Ct20. Western blots were repeated three times with similar results. (C–E) Immunostaining for the B-subunit of V-ATPase in longitudinal sections of UVDs dissected every 4 h in LD (C), DD (D) or constant light (LL) conditions (E). Each picture shows representative columnar epithelial cells with their apical sides on the left and blue-gray elongated nuclei. Red V-ATPase signals appear to be concentrated in small vesicle-like structures. A robust increase in the amount of V-ATPase at Zt16–20 is evident in LD and in the second cycle in DD conditions; however, the amount of protein does not show a daily rhythm in LL conditions. The experiment was performed on six males per time point in each photoregime with similar results. No staining was detected in control tissues treated with antigen-preabsorbed antibody. Scale bar, 20 μm. Horizontal bars represent day (open), night (filled) and former day (hatched) portions of the photo-regime.

of the reaction. The levels of V-ATPase were low during the day at Zt4 and Zt8, began to increase at Zt12 and were high during the night at Zt16–20. The rhythm in protein abundance appeared to persist on the second day after the moths had been transferred into DD conditions; relatively less V-ATPase was detected during the subjective day at circadian time 8 (Ct8) than during the night at Ct20 (Fig. 3B). The fact that more protein was seen at Ct8 in DD conditions than at Zt8 in LD conditions may reflect the fact that circadian rhythms tend to decrease in amplitude in free-running conditions.

To confirm the above results and to determine the spatial expression of the proton pump, we performed an

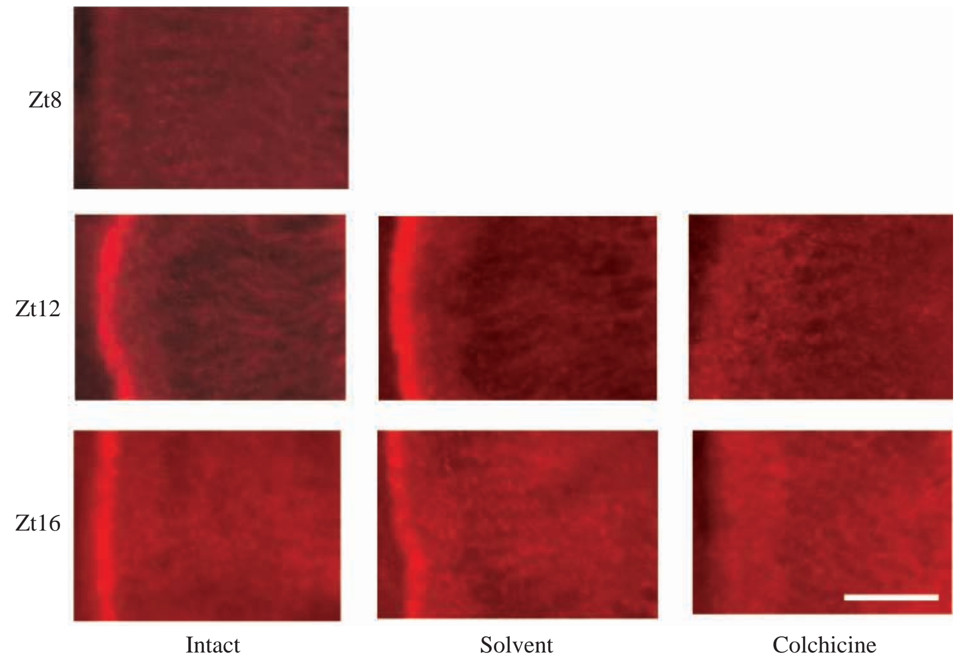

Fig. 4. Localization of V-ATPase in the upper vas deferens (UVD) of intact, solvent-injected and colchicine-injected moths. Injections were delivered at Zeitgeber time 8 (Zt8), when moths have low levels of V-ATPase. Colchicine inhibited apical accumulation of V-ATPase in UVDs dissected at Zt12 and Zt16. V-ATPase was detected with an antibody against its B-subunit followed by Texas-Red-conjugated secondary antibody. The images shown are representative of six UVDs processed after each treatment at each time point. Scale bar, 20 μm.

immunocytochemical analysis with the antibody against the B-subunit of V-ATPase on reproductive tracts from moths kept in LD, DD and LL conditions. Very low levels of V-ATPase were detected in the testes and seminal vesicles of LD moths at all times of day (data not shown). However, there was a robust daily rhythm in the levels and subcellular localization of V-ATPase in all the cells forming the columnar epithelium that surrounds the UVD lumen (Fig. 3C). During the day, at Zt4–8, the V-ATPase signal was low in the UVD epithelium. The level of immunostaining increased significantly at Zt12, and its punctuated occurrence suggested localization in small vesicles, which seemed to be more abundant in the apical portion of each cell. During the middle of the night (Zt16), a massive accumulation of V-ATPase occurred in the apical portions of epithelial cells. At Zt20–24, V-ATPase was still abundant, but more evenly spread between the apical and basal regions. A very similar rhythmic pattern of V-ATPase expression persisted in UVDs taken from moths kept for 2 days in DD conditions (Fig. 3D), demonstrating that it is an endogenous circadian rhythm rather than a direct response of the cells to LD cycles.

In LL conditions, the levels of V-ATPase were low at all time points tested (Fig. 3E). In both LD and DD conditions, there was a clear correlation between the highest concentration of apical V-ATPase and the lowest pH in the UVD lumen, which occurred at Zt16 in LD and DD conditions (compare Figs 2 and 3). Both the rhythm of pH and the rhythm of V-ATPase abundance were abolished in LL conditions, which disrupt circadian clocks in insects (Saunders, 1982). This suggests that the rhythm in pH is generated by a clock-controlled rhythm in V-ATPase.

Causal relationship between rhythms in pH and V-ATPase activity

To test the relationship between the decline in luminal pH and the accumulation of V-ATPase at the apical cell surface, we attempted to prevent this accumulation by injecting the microtubule-disrupting drug colchicine. Moths were injected at Zt8, when V-ATPase levels are low. In the intact and solvent-injected moths, a clear increase in apically located V-ATPase was evident at Zt12 (Fig. 4), i.e. somewhat earlier than when a less-sensitive detection method was used (compare Fig. 3). In colchicine-injected males, the apical accumulation of V-ATPase was prevented at Zt12 and partially inhibited at Zt16, although the overall levels of cellular V-ATPase had evidently increased by Zt16 (Fig. 4). We subsequently examined luminal pH in males that had been treated with colchicine at Zt8 and dissected at Zt12. In solvent-injected males, the pH declined significantly by Zt12, as in intact males (Fig. 5). However, such a decline did not occur in colchicine-injected males. Thus, apical translocation of V-ATPase, which is required for its fusion with the cell membrane, is necessary for the acidification of the UVD lumen.

The involvement of V-ATPase in the acidification of the UVD lumen was confirmed by demonstrating that bafilomycin, a specific inhibitor of this enzyme (Gagliardi et al., 1999), prevents the decline in pH. We injected bafilomycin into the UVDs at Zt8 and measured luminal pH 8 h later, at Zt16 (Fig. 6). The initial pH at Zt8 was in the range 6.8–7.0 in all the moths examined. By Zt16, the pH had dropped significantly in the intact and solvent-injected UVDs. In contrast, the pH remained high at Zt16 in the bafilomycin-injected UVDs, demonstrating that the acidification of the UVD lumen is caused by the activity of V-ATPase.

Discussion

Our data demonstrate a robust circadian rhythm of luminal pH in the vas deferens of *S. littoralis*. Luminal acidification

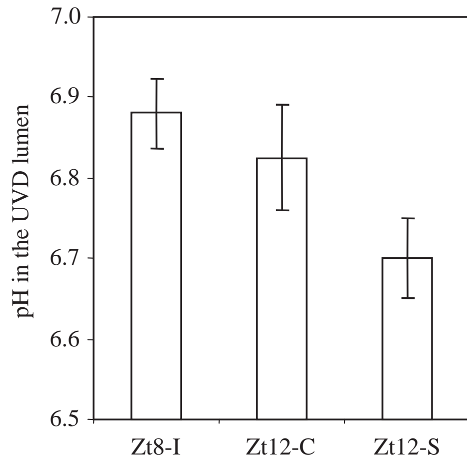

Fig. 5. Effects of colchicine on luminal pH. The columns show luminal pH in the upper vas deferens (UVD) of intact moths at Zeitgeber time 8 (Zt8-I), and in moths that were injected at Zt8 and evaluated at Zt12 after colchicine injection (Zt12-C) or solvent injection (Zt12-S). pH remained high in colchicine-injected moths, but declined significantly (Student's *t*-test; $P<0.05$) in solvent-injected moths. Each column represents the pH (mean ± S.E.M.) for 5–6 individuals per treatment.

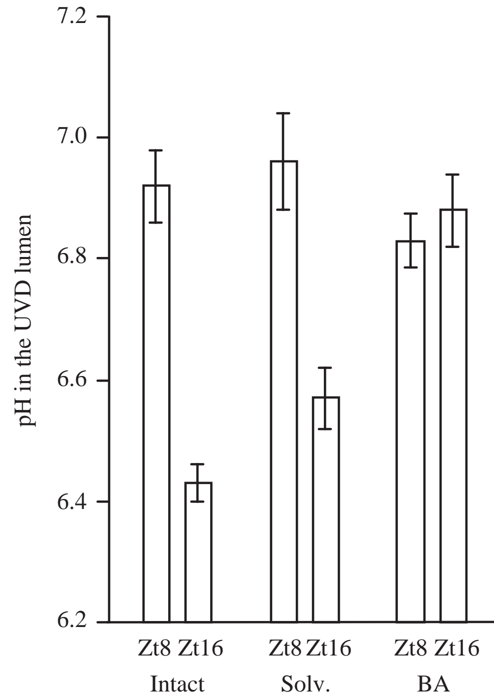

Fig. 6. Effects of bafilomycin (BA) on the pH in the lumen of the upper vas deferens (UVD). UVDs were injected at Zeitgeber time 8 (Zt8) with bafilomycin or 6% dimethylsulfoxide (Solv.) or left untreated (Intact). The pH was measured at Zt8 in groups of control moths and at Zt16 in injected moths. Bafilomycin prevented the decline in pH relative to the beginning of the experiment at Zt8, while solvent-injected and intact UVDs showed a statistically significant (Student's *t*-test; $P<0.01$) decline in pH. Each column represents the pH (mean ± S.E.M.) for eight individuals per treatment.

coincides with the accumulation of sperm released from the testis into the UVD a few hours earlier (Fig. 1). However, the decline in pH is not triggered by the presence of sperm since the pH rhythm is detected in UVDs of young animals prior to first sperm release (P. Bebas and J. M. Giebultowicz, unpublished observations). What is the function of the pH rhythm in the insect vas deferens? Acidification coincides with the daily peak of glycoprotein secretion from the UVD epithelium (Riemann and Giebultowicz, 1991) (P. Bebas, unpublished observations). Glycoproteins play a major role in the maturation of spermatozoa (Kirchhoff et al., 1997), and acidic pH may provide an optimal environment for the interaction between glycoproteins and spermatozoa. We hypothesize that the acidification rhythm is essential for the coordinated maturation of spermatozoa. This is supported by observations that constant light conditions, which disrupt rhythms associated with sperm release (Giebultowicz et al., 1988, 1996) and the luminal pH rhythm (present study), lead to male sterility (Giebultowicz et al., 1990; Bebas and Cymborowski, 1999). The importance of the acidic pH in the sperm ducts has previously been suggested in mammals. Luminal acidification occurs in the rat epididymis and vas deferens (Levine and Marsh, 1971), and it has been proposed that a low pH is required for the maturation of spermatozoa and for the inhibition of precocious sperm motility (Hinton and Palladino, 1995). Our data add a novel layer of circadian regulation to the acidification process in the insect vas deferens and raise the interesting possibility that similar regulatory mechanisms may operate in mammals, since circadian clock genes are expressed in the reproductive tissues of male rats (Tei et al., 1997; Zylka et al., 1998).

Circadian rhythms in pH have been reported previously in the unicellular organism *Gonyaulax* (Roenneberg and Merrow, 1999) and in the goldfish and rabbit retinas (Dmitriev and Mangel, 2000, 2001). However, the molecular and cellular bases of these rhythms have not been investigated. Here, we present several lines of evidence suggesting that periodic luminal acidification of the insect UVD is achieved by the daily rhythm in abundance and apical targeting of the proton pump, V-ATPase. First, the pH rhythm is abolished in constant light, which disrupts the V-ATPase rhythm. Second, the decline in luminal pH is prevented when the apical targeting of V-ATPase is disrupted by specific inhibitors of V-ATPase activity. V-ATPase is also involved in the acidification of mammalian seminal ducts. A population of V-ATPase-rich cells has been detected in the epididymis and vas deferens of rats, and proton secretion in these tissues has been demonstrated directly (Brown et al., 1997; Brown and Breton, 2000). An interesting difference between moths and rats is that, in the moth, all UVD epithelial cells express V-ATPase, whereas in rats only approximately 40% of vas deferens epithelial cells are rich in V-ATPase (Breton et al., 1996). Nevertheless, the molecular underpinnings of seminal duct acidification appear to be conserved from insects to mammals.

Our data strongly suggest that V-ATPase in the UVD

epithelium is regulated by the circadian clock. V-ATPase is a multi-subunit proton pump composed of two structural domains, a peripheral catalytic V_1 sector and a transmembrane, proton-conducting V_o sector. We detected a clear daily rhythm in the abundance of the B-subunit of the V-ATPase. The B-subunit is an ATP-binding, regulatory component of the catalytic V_1 sector of the holoenzyme. It remains to be investigated whether other subunits of this proton pump also undergo rhythmic changes at the protein level and whether the circadian clock transcriptionally regulates genes coding for different V-ATPase subunits. V-ATPases are highly conserved enzymes providing the proton-motive force for numerous secondary transport processes (Dow et al., 1997; Nelson and Harvey, 1999), in addition to regulating endosomal and extracellular pH. V-ATPases are involved in the release of secretory proteins (Schoonderwoert et al., 2000), in the recycling of synaptic vesicles (Cousin and Nicholls, 1997) and in the transport of ions and fluids across the plasma membrane in various epithelia (Harvey et al., 1998). V-ATPases are found in brain, kidney, osteoclasts and cancer cells (Wieczorek et al., 1999). Given their multitude of functions and locations, the expression and activity of V-ATPases are expected to be regulated in various ways (Merzendorfer et al., 1997). We demonstrate here, for the first time, that the circadian clock is a key factor in regulating V-ATPase levels in insect vas deferens; it remains to be determined whether V-ATPase levels change rhythmically in other systems.

The daily rhythm in the apical targeting of V-ATPase within the epithelial cells represents a novel output of the clock at the cellular level. We observed that V-ATPase was uniformly distributed throughout the cells during the day and then accumulated in the apical region early in the night, concurrently with the decline in luminal pH. It is known that V-ATPase is inserted into the membranes of specialized intracellular acidic vesicles and may be recycled between these vesicles and the plasma membrane; the exocytotic fusion of V-ATPase vesicles with the plasma membrane enables protons to move to the extracellular space (Wieczorek et al., 2000). We infer that similar mechanisms may operate in the moth UVD epithelium; the punctuated appearance of the V-ATPase signal in our immunocytochemical analysis (Fig. 3) suggests that V-ATPase is vesicle-bound in this tissue. We also demonstrated that the migration of V-ATPase towards the apical plasma membrane is impaired by a microtubule-disrupting drug, which is consistent with the previously documented involvement of microtubules in apical targeting of this enzyme (Brown et al., 1997).

The mechanism by which the circadian system generates molecular and cellular rhythms in the UVD epithelium remains to be investigated. The clock gene *period*, which is essential for circadian clock function, is rhythmically expressed in all UVD epithelial cells of moths (Gvakharia et al., 2000), including *S. littoralis* (P. Bebas, unpublished observations), suggesting that the clock mechanism operates ubiquitously in the UVD cells and may be controlling output rhythms at the level of a single cell. However, intercellular communication may also play a role in synchronizing the activity of UVD epithelial cells. Taken together, our results provide novel insights into the organization of a peripheral circadian system by demonstrating that the cellular rhythms in V-ATPase abundance and its apical targeting, generated at the single-cell level, lead to a diurnal change in pH at the organ level.

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