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Phase Shifts of the Circadian Locomotor Rhythm Induced by Pigment-Dispersing Factor in the Cricket *Gryllus bimaculatus*

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ABSTRACT—Pigment-dispersing factors (PDFs) are octadeca-peptides widely distributed in insect optic lobes and brain. In this study, we have purified PDF and determined its amino acid sequence in the cricket *Gryllus bimaculatus*. Its primary structure was NSEIINSLLGLPKVLNDA-NH_2, homologous to other PDH family members so far reported. When injected into the optic lobe of experimentally blinded adult male crickets, *Gryllus*-PDF induced phase shifts in their activity rhythms in a phase dependent and dose dependent manner. The resulted phase response curve (PRC) showed delays during the late subjective night to early subjective day and advances during the mid subjective day to mid subjective night. The PRC was different in shape from those for light, serotonin and temperature. These results suggest that PDF plays a role in phase regulation of the circadian clock through a separate pathway from those of other known phase regulating agents.

Key words: pigment-dispersing factor, circadian rhythm, crickets, phase shifts, phase response curve

INTRODUCTION

Pigment-dispersing hormones (PDHs) are octadeca-peptides that are secreted from the sinus gland in the eyestalks, translocate retinal distal pigments and disperse epithelial chromatophoral pigments in crustaceans (Rao and Riehm, 1993). Some of them have been purified and their primary structures were determined in several species (Rao and Riehm, 1989, 1993). Related peptides were also found in some insect species (Park and Hall, 1998; Rao and Riehm, 1993). The primary structures show high similarity in amino acid sequence between crustaceans and insects. In insects, these peptides are designated as pigment-dispersing factors (PDFs), since their physiological roles have not yet been clearly defined (Rao and Riehm, 1993). Several lines of evidence suggest that PDF is involved in the regulation of circadian rhythms in some insects. Immunocytochemistry has revealed that in many insect species, PDF was localized in the optic lobes, the circadian pacemaker region, and in the brain (Helfrich-Förster and Homberg, 1993; Nässel *et al*., 1993; Okamoto *et al*., 2001). In *Drosophila,* some of the PDF-containing neurons were shown to also express PERIOD protein, a principal clock component (Helfrich-Förster, 1995), and pdf^0 mutant flies lacking functional PDF often became arrhythmic in constant darkness, suggesting that PDF is an output signal of the circadian clock (Renn *et al*., 1999). Physiological effects of PDF on the circadian system have been examined in the cockroach, *Leucophaea maderae*. Petri and Stengl (1997) showed that application of PDF in the optic lobe shifted the phase of locomotor activity rhythm, and the phase response curve for PDF differed from that for light. It was thus suggested that PDF was not in the photic entrainment pathway in the cockroach. However, they used the PDF of the cricket *Acheta domesticus*, thus it is necessary to examine the effects of conspecific PDF on the rhythm to clarify its authentic physiological role in the circadian system.

In the present study, we investigated the functional role of conspecific PDF in the circadian system of the cricket

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Gryllus bimaculatus. The circadian clock of this insect is located one in each of the paired optic lobes (Tomioka and Chiba, 1984, 1992). The two clocks are mutually coupled to move in synchrony by exchanging coupling signals through a neural pathway (Tomioka *et al.*, 1991; Tomioka, 1993; Yukizane and Tomioka, 1995). Immunocytochemistry with anti-*Uca*-β-PDH antibody revealed that there were widely distributed PDH immunoreactive neurons in the optic lobe. They were composed of two groups, one located in the proximal medulla and the other near lamina (Okamoto *et al.*, 2001). As the first step, we have purified the PDF from the cricket brain and determined its primary structure, then synthesized *Gryllus*-PDF. We have then tested the effects of *Gryllus*-PDF on the circadian locomotor rhythm. The resulted phase response curve for PDF was quite different from those obtained for light, serotonin and temperature, suggesting that PDF has a role in phase regulation of the circadian clock, but it acts through a different pathway from those of other phase-shifting agents.

MATERIALS AND METHODS

Experimental animals

All experiments were performed with adult male crickets, *Gryllus bimaculatus,* obtained from laboratory colonies maintained at a constant temperature of 25±0.5°C and a 12 hr light: 12 hr dark cycle (light: 0600 to 1800 hr; Japanese Standard Time) with a continuous supply of food (CA-1, Nihon Clea) and water.

Procedure of purification

The cerebral ganglia excised from 200 specimens of adult crickets were immediately frozen in liquid nitrogen. The frozen ganglia were boiled in water (15 ml) for 5 min to inactivate proteolytic enzymes. After cooling, acetic acid was added to the water (final concentration; 3%). The tissue was homogenized first by Polytron homogenizer for 5 min and then by a Teflon-glass homogenizer for 5 min on ice. The homogenate was centrifuged at 15,000 g and at 4°C for 20 min, and the supernatant was collected. The precipitate was extracted again with 10 ml of 3% acetic acid. The collected supernatants were loaded onto a C18 reversed-phase cartridge (Sep-Pak C18, Waters). The cartridge was washed with 10% acetonitrile/0.1% trifluoroacetic acid (TFA), and then eluted with 50% acetonitrile/0.1% TFA. The obtained fraction was concentrated to a small volume and subjected to HPLC purification. First, the extract was separated on a C18 reversed-phase column (Capcell Pak C18, 10×250 mm, Shiseido) with a linear gradient of acetonitrile (0–60% in 60 min) in 0.1% TFA at a flow rate of 1 ml/min. The chromatography (also the following chromatographies) was monitored at 220 nm. Fractions of 1 ml each were collected, and an aliquot was applied to a competitive enzyme-linked immunosorbent assay (ELISA) using anti-β-PDH antiserum as described below. An immunoreactive fraction eluted at 38-39% acetonitrile was obtained. The peak was next loaded on a cation-exchange column (TSKgel SP-5PW, 7.5×75 mm, Tosoh), and the flowthrough fraction showed immunoreactivity against anti-β-PDH. The flowthrough was injected into a reversed-phase column (Capcell Pak C18, 4.6×150 mm, Shiseido) which was developed by a linear gradient of 25–45% acetonitrile in 0.1% TFA. Fractions of 1ml each was collected and an aliquot were applied to a competitive ELISA. An immunopositive fraction eluted at 32% acetonitrile was obtained. The immunopositive fraction was further purified on the same column by the isocratic elution with 32% acetonitrile/0.1% TFA.

Competitive ELISA

The method of competitive ELISA was principally the same as described for the molluscan neuropeptide (Fujisawa, 1996). *Acheta*-PDF (Rao and Riehm, 1989) was coated on a 96-well ELISA plate (#25801, Corning) at a concentration of 1.5×10^{-8} M at 37°C for 2 hr. Blocking solution containing 1% bovine serum albumin (BSA)/10 mM Na-phosphate buffer/150 mM NaCl (10 mM PBS, pH 7.5) was added to the plate at 37°C for 1 hr. After washing with 0.2% Tween-20/10 mM PBS three times, the plate was incubated with the anti-β-PDH antiserum (1:20,000) and either a standard peptide, *Acheta*-PDF, or an aliquot of HPLC fractions at 4°C for overnight. Following the wash as described above, the plate was incubated with the alkaline-phosphatase labeled secondary antibody (Vector Laboratories Inc.) at 37°C for 1 hr. Finally the enzyme substrate p-nitrophenyl phosphate was added, and the coloring reaction proceeded at room temperature for 1 hr. OD at 405 nm was read on a microtitre plate reader (Applied Biosystems).

Structural analysis

The purified peptide was subjected to amino acid sequence analysis by Edman degradation method on an automated gas-phase sequencer (PSQ-1, Shimadzu). Molecular weight of the peptide was measured by matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on Voyager Elite (Perseptive Biosystems).

Activity recording

The crickets were kept individually in an activity chamber of transparent plastic box (20×7×6 cm) with a tilting substratum. Locomotor activity was monitored using a magnetic reed switch connected to a computer. Signals produced by the switch by a moving animal on the tilting plate were collected by the computer, which summed a total number of signals for every 6 min and stored it on a hard disk. Food and water were available *ad libitum*. The activity chamber was placed in an environment-controlled room in which the temperature was kept constant at 25±0.5°C. The experiments were performed in continuous light conditions (LL), which were made by a 20 W cool white fluorescent lamp. The light intensity at the activity chamber was about 120 lx.

Surgery

Experimentally blinded animals were prepared by unilateral removal of the lamina-medulla complex, which hereafter will be referred to as the 'optic lobe' for convenience, covering the contralateral compound eye with a piece of aluminium foil, then painting the covered area with black coating. Surgical removal of the optic lobe was performed under CO_2 anesthesia as follows. An animal was fixed on a specially designed plastic platform to immobilize its head. The cuticle around the compound eye was cut with a fine razor knife, and the eye was pried open with forceps to expose the optic lobe. The lobe was then removed by cutting both the optic nerve and the optic stalk connecting the medulla and the lobula with a pair of micro-scissors. After the surgery, the compound eye was replaced and the wound was sealed naturally with clotted hemolymph. Before releasing into the activity chamber, either of the forewings was removed to prevent any interindividual sound communication. At the end of the experiment, the head was carefully dissected to verify the success of the surgical lesion under a dissecting microscope.

Microinjection

Injections were performed into the optic lobe through the compound eye using a nanoliter injector (WPI, A20XVY), which was equipped with a glass micropipette and mounted on a micromanipulator (Narishige, M-3333). *Gryllus*-PDF was dissolved in an insect Ringer's solution (Fielden, 1960) at the desired concentrations. PDF (22 nl of 0.1 mM) was injected at various circadian times (CTs)

to obtain a phase response curve (PRC). CT 0 and CT 18 were selected as maximal responsive zones of the PRC to study dose responses. Tips of the micropipette were checked before and after the injection in order to ensure that the injection was successfully performed. The volume of injected solution was estimated by measuring the diameter of droplets injected into mineral oil under a microscope. The volume for a single injection was 22.18±3.37 nl (mean±SD). The same volume of Ringer's solution was injected as a control. Diffusion of the microinjected material into the optic lobe was confirmed by injecting 10% green food dye dissolved in Ringer's solution. Within 30 min, it diffused to near the proximal medulla.

Data analysis

Event records for locomotor activity were double plotted by computer with a resolution of six minutes. Onsets of activity designated as CT 12 were chosen as the phase reference point of the rhythm, since they were the most stable and reliable point in the free-running state of adult crickets (Okada *et al.*, 1991). Free-running periods and phases were determined by fitting lines by eye through activity onsets in a steady free-running state, one before and one after the injection. Each line fitting was performed through at least 7 days of data. The magnitude of the phase shift was determined as the interval between the lines extrapolated to the day of the treatment. Phase shifts induced by PDF or vehicle were pooled in two-hour bins and plotted against various CTs for construction of PRC. Statistical significance of the results was determined by Student's *t*-test.

RESULTS

Isolation and characterization of the *Gryllus*-PDF

The *Gryllus*-PDF was obtained from extracts of cerebral ganglia by a combination of HPLC and competitive ELISA using anti-*Uca*-β-PDH antibody. Acetic acid extracts of cricket brains were passed through C18 reversed phase cartridge. The retained material eluted with 50% acetonitrile/0.1% TFA was then subjected to the first step of HPLC purification with a C18 reversed phase column with a linear gradient of 0–60% acetonitrile. An immunoreactive fraction was obtained at 38–39% acetonitrile. The PDF immunoreactive fraction was subjected to a cation-exchange column, and the obtained flowthrough fraction with immunoreactivity against anti-β-PDH was further subjected to reversed-phase HPLC purification under a linear gradient of 25–45% acetonitrile. An immunopositive fraction was detected at 32% acetonitrile (Fig. 1). The immunopositive fraction was further purified on the same column by an isocratic elution with 32% acetonitrile/0.1% TFA. Fig. 2 shows the HPLC chromatogram of the final step of the purification of *Gryllus*-PDF on a reversed-phase column. Amino acid sequence analysis of *Gryllus*-PDF revealed that its sequence was NSEIINSLLGLPKVLNDA. The MALDI-TOF-MS analysis showed

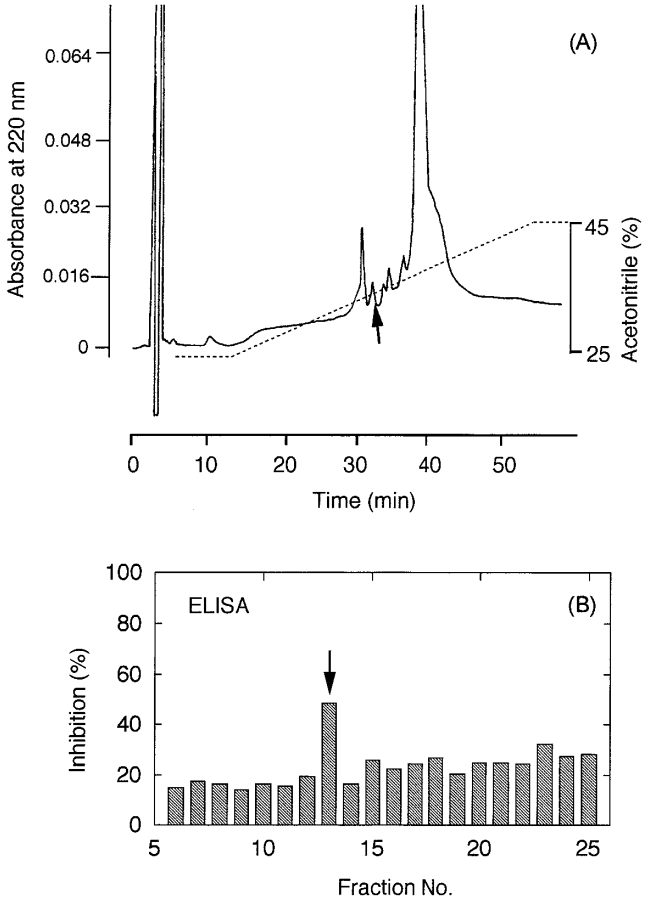

Fig. 1. Profile of the 2nd step of HPLC purification of the *Gryllus*-PDF on a reversed-phase column (A) and results of competitive ELISA (B). The elution was performed with a 40 min linear gradient of 25–45% of acetonitrile in 0.1% TFA (pH 2.2). The PDF activity was detected in fraction No. 13 (arrows) by competitive ELISA.

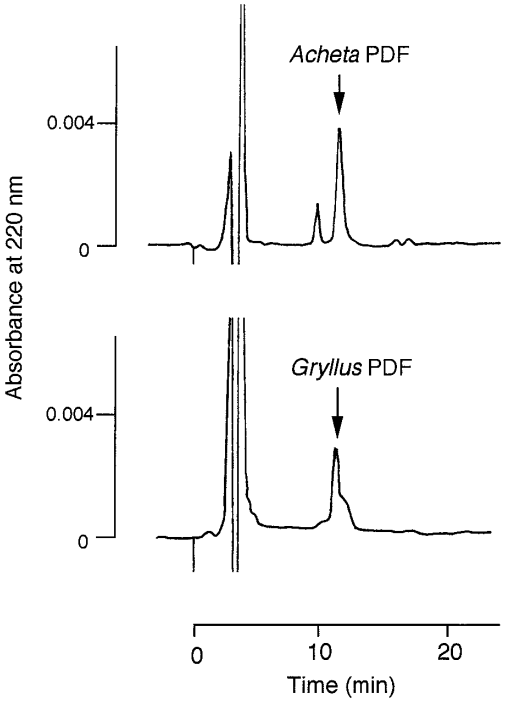

Fig. 2. Profile of the final step of HPLC purification of *Gryllus*-PDF on a reversed-phase column (bottom panel). The elution was performed isocratically with 32% acetonitrile in 0.1% TFA. The peak occurred at exactly same retention time as synthesized *Acheta*-PDF (top panel).

Table 1. Comparisons of the primary structures of the crustacean pigment-dispersing hormones (PDHs) and insect pigment-dispersing factors (PDFs).

Species	Amino acid sequence	References
Crustacean (PDH)		
Uca pugilator	NSELINSILGLPKVMNDA-NH_2	(Rao *et al*., 1985)
Cancer magister	NSELINSILGLPKVMNDA-NH_2	(Kleinholz *et al*., 1986)
Callinectes sapidus	NSELINSILGLPKVMNDA-NH_2	(Mohrherr *et al*., 1990)
Orconectes immunis	NSELINSILGLPKVMNEA-NH_2	(Rao and Riehm, 1993)
Penaeus aztecus	NSELINSLLGIPKVMNDA-NH_2	(Rao and Riehm, 1993)
Pandalus borealis	NSGMINSILGIPRVMTEA-NH_2	(Rao and Riehm, 1993)
Insect (PDF)		
Drosophila melanogaster	NSELINSLLSLPKNMNDA-NH_2	(Park and Hall, 1998)
Periplaneta americana	NSELINSLLGLPKVLNDA-NH_2	(Rao and Riehm, 1993)
Romalea microptera	NSEIINSLLGLPKLLNDA-NH_2	(Rao *et al*., 1987)
Acheta domesticus	NSEIINSLLGLPKVLNDA-NH_2	(Rao and Riehm, 1988)
Gryllus bimaculatus	NSEIINSLLGLPKVLNDA-NH_2	

that the molecular weight of the peptide was 1908.04. From these results, the structure of the peptide was considered to be NSEIINSLLGLPKVLNDA-NH_2. The structure is homologous to other PDH family members so far reported (Rao and Riehm, 1993; Park and Hall, 1998) and it is the same of *Acheta*-PDF, suggesting the structural conservation among cricket species (Table 1).

Phase shifts induced by PDF

All the crickets with the optic lobe unilaterally removed and with the contralateral compound eye occluded showed a stable free-running activity rhythm in LL. The free-running

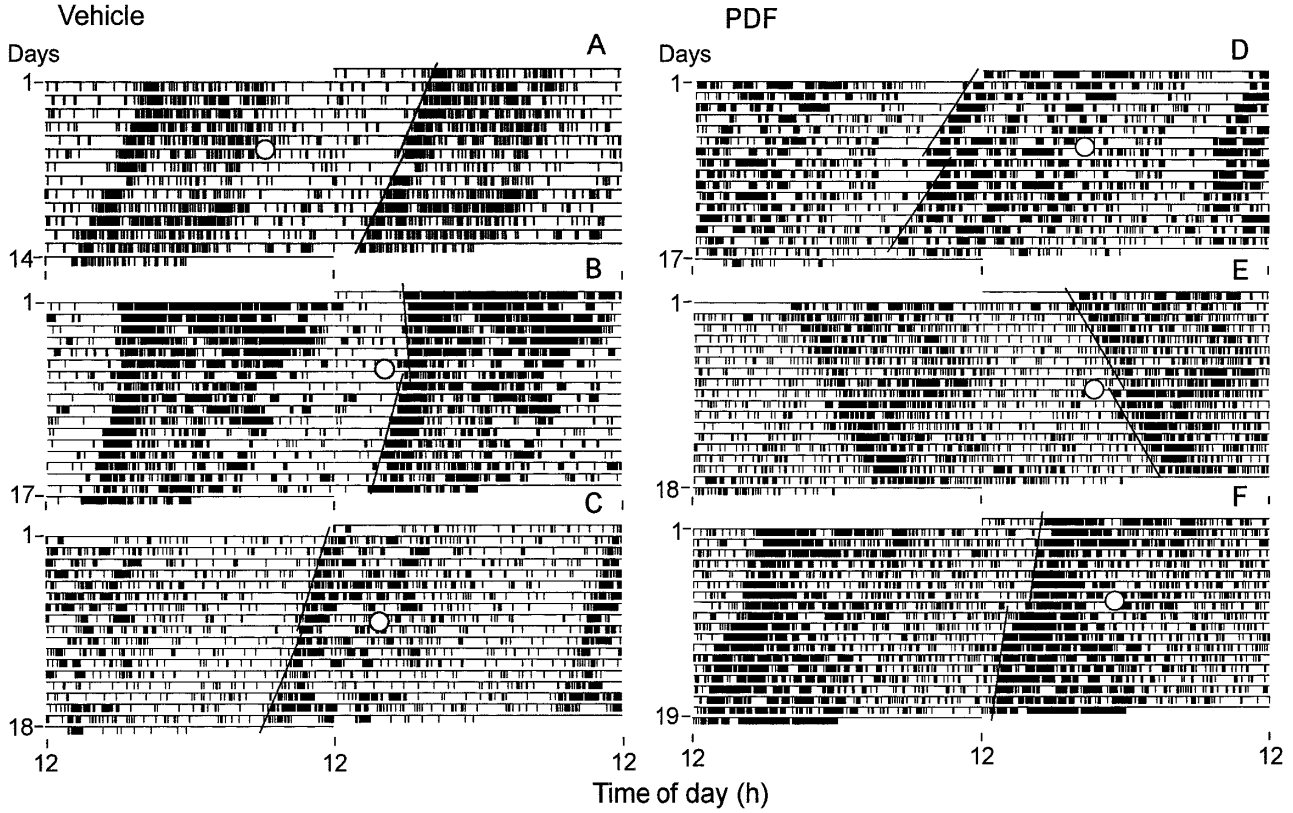

Fig. 3. Representative double plotted locomotor activity records of crickets *G. bimaculatus*, showing phase shifting effects of 22 nl of vehicle (Ringer's solution, A–C) or PDF (0.1 mM) (D–F). Injection was performed at CT 0 (A, D), 10 (B, E) and 18 (C, F). PDF injection caused phase delay at CT 0 (D) and phase advance at CT 10 (E) and CT 18 (F), whereas vehicle injection did not cause robust shifts. Open circles indicate the time of microinjection. Oblique lines show eye-fitted lines through activity onsets.

period of the experimentally blinded animals was 24.21± 0.55 hr (mean±SD; n=172) with a range of 22.72 hr to 25.65 hr. The relatively large inter-individual variation might be attributable to a slim fraction of light penetrating through head cuticle and perceived by the occluded compound eye. Nevertheless, the stability of the free-running rhythm enabled us to examine phase-shifting effects of PDF under these conditions.

Crickets administered with *Gryllus*-PDF (22 nl of 0.1 mM) showed significant phase shifts of the circadian locomotor rhythm when compared to those treated with vehicle at the same CTs. Vehicle injections induced small phase shifts of less than 1 hr, and the direction of the shifts was inconsistent (Figs. 3A–C and 4A). Fig. 3D–F exemplifies effects of PDF injection on the locomotor rhythm. PDF induced phase shifts in a phase dependent manner: it phase delayed the rhythm at CT 0 whereas advanced at CT 10 and 18. Phase advances often took several transient cycles (Fig. 3F). The resulted PRC is shown in Fig. 4B. PDF induced advance phase shifts during the mid subjective day to the mid subjective night (CT 6 to CT 18) with a maximal advance of 2.05±0.42 hr (mean±SEM) at CT 18, whereas it elicited delay shifts during the late subjective night to the early subjective day (CT 20 to CT 4) with a maximal delay of –1.83±0.28 hr (mean±SEM) at CT 0. The phase shifts induced by PDF were statistically significant when compared with those induced by the vehicle (*t*-test, $P<0.05$), except for CTs 6, 8 and 10 (*t*-test, $P>0.05$).

The PDF administration frequently altered the free-running period, with maximal shortening by 0.52±0.20 hr (mean ±SEM) at CT 6 and maximal lengthening by 0.62±0.28 hr (mean±SEM) at CT 2. However, the alterations were not significantly different from those injected with the vehicle (*t*-test, $P>0.05$). Further analysis of the PDF induced period responses showed that there was no correlation between the period alteration and the direction and magnitude of the phase shifts. The amount of activity was also analyzed for 3 hr following the injection of PDF or vehicle. Statistically significant enhancement of activity by PDF was observed only at CT 4 (*t*-test, $P<0.05$).

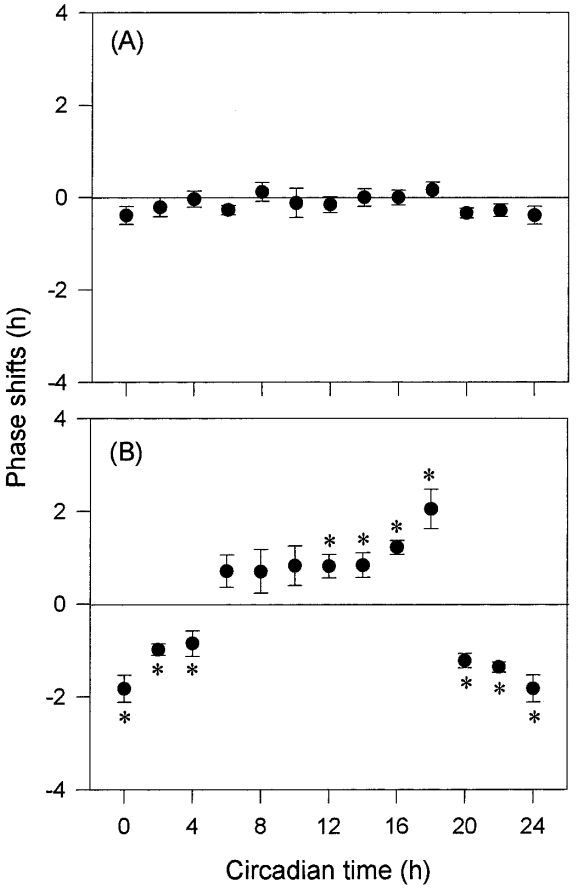

Fig. 4. Phase response curves induced by a single administration of vehicle (A) or 0.1 mM PDF (B) in the cricket *G. bimaculatus.* Data are pooled in 2 hr bins and mean±SEM is plotted against CT when injection was made. Asterisks indicate that the magnitude of phase shifts by PDF are significantly greater than that induced by vehicle injections at the same CT ($P<0.05$, *t*-test). Advance phase shifts are plotted as positive (+) value and delay shifts as negative (–).

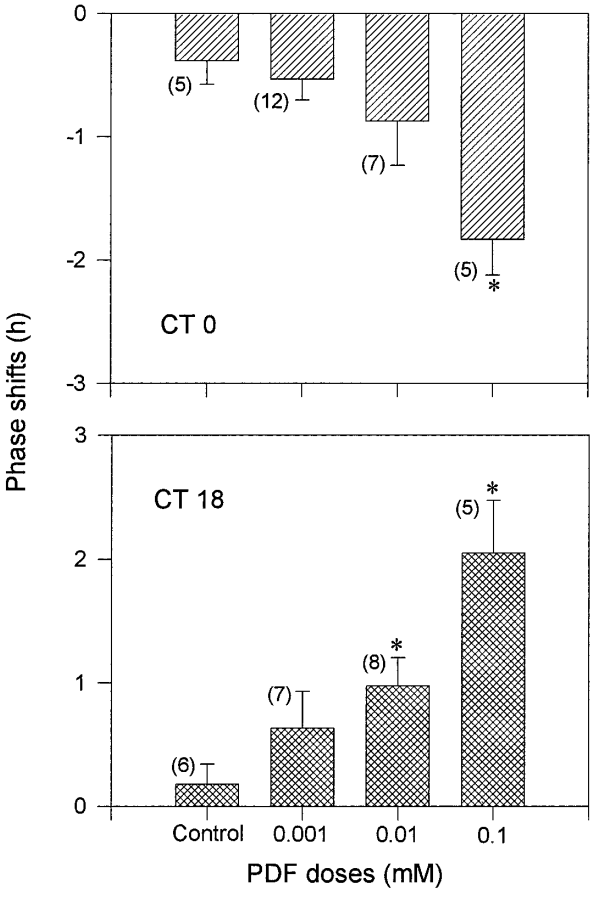

Fig. 5. Phase shifts (mean±SEM) induced by a single administration of vehicle (Ringer's solution) or various doses of PDF at CT 0 (upper panel) and CT 18 (lower panel) in the cricket *G. bimaculatus*. Asterisks indicate that the phase shifts are significantly greater than that induced by vehicle injection ($P<0.05$, *t*-test). Numbers in parenthesis indicate the number of animals used.

Dose dependency of phase shifts

To examine dose dependency of the phase shifting effect of PDF, different doses of PDF (0.001 mM, 0.01 mM and 0.1 mM) were injected into the optic lobe at CT 0 and CT 18 at which the maximal delay and advance shifts were induced, respectively. The phase shifting effect of PDF was clearly dose-dependent at both CTs (Fig. 5). The magnitude of both phase delays and advances increased with increasing the dose of PDF. However, significant phase shifts were observed at 0.1 mM at CT 0 (*t*-test, $P<0.05$) and 0.01 and 0.1 mM at CT18 (*t*-test, $P<0.05$).

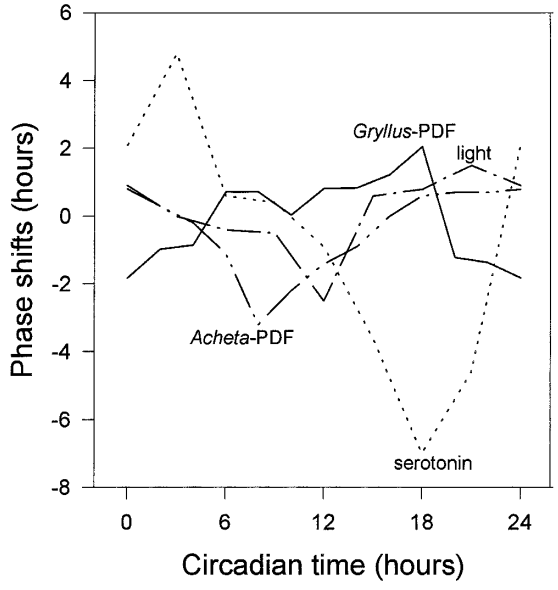

Fig. 6. Phase response curves for light, serotonin and *Gryllus*-PDF in the cricket *Gryllus bimaculatus*, and for *Acheta*-PDF in the cockroach *Leucophaea maderae*. The cricket phase response curve for *Gryllus*-PDF is different from those obtained for light and serotonin (Tomioka, 1999) and also from that for PDF in the cockroach (Petri and Stengl, 1997).

DISCUSSION

Chemistry of *Gryllus*-PDF

The results of the present study determined the primary structure of the *Gryllus*-PDF to be NSEIINSLLGLPKVLNDA-NH_2, as predicted from the result of molecular cloning of *pdf*-gene (Chuman *et al.*, 2002), by a combination of amino acid sequence analysis and molecular weight analysis of the extracted PDF. The primary structure is identical with that of *A. domesticus* and exhibits high similarity to those of other insect species (Table 1). Only a sole residue substitution was found for PDFs of *Periplaneta americana* and *Romalea microptera*, i.e., Leu/Ile^4 and Leu/Val^{14}, respectively. However, in comparison with *Drosophila*-PDF, four residue substitutions were found, i.e., Leu/Ile^4, Ser/Gly^{10}, Asn/Val^{14}, and Met/Leu^{15}. The *Gryllus*-PDF also has high similarity to those of crustacean PDHs (Table 1). Especially for *Uca*-β-PDH, only three residue substitutions were seen, i.e., Leu/Ile^4, Ile/Leu^8, and Met/Leu^{15}.

Functional role of PDF

Although phase shifting effects of PDF has been reported for cockroaches (Petri and Stengl, 1997), the present study demonstrated for the first time the phase shifting effects of conspecific PDF on the circadian activity rhythm in the cricket *G. bimaculatus*. The PRC resulted by PDF injection showed advances during the mid subjective day to mid subjective night and delays during the late subjective night to early subjective day, suggesting that it may play a role in phasing the circadian clock. The PRC is quite different from that for light (Okada *et al.*, 1991; Tomioka, 1999): PDF advances the clock when light has no effect or induces delays, while it delays the clock when light induces advances or has no robust effect (Fig. 6). The fact suggests that PDF neurons may not serve as a photic input pathway to the circadian clock like in cockroaches (Petri and Stengl, 1997). The PRC also differs from that for serotonin (Fig. 6), where the maximal delay region occurred at the mid subjective night (Tomioka, 1999), when PDF caused the maximal advance. It also differs from those for temperature step-up and –down, where phase advances and delays are mostly induced by step-up and –down, respectively, at all CTs (Ikeda and Tomioka, 1993). It is thus suggested that PDF acts on the circadian pacemaker through a different pathway from those for light, serotonin and temperature. In cockroaches PDF is suggested to be involved in the coupling between the two optic lobe clocks (Stengl and Homberg, 1994; Petri and Stengl, 1997). However, this is uncertain in crickets, since the shape of the PRC is quite different from the shape of the period modulation curve derived from the mutual interaction of the clocks (Tomioka *et al.*, 1991; Tomioka, 1993).

There are lines of evidence that enhanced activity often causes phase shifts through its feedback to the circadian clock in mammalian species (Mrosovsky and Salmon, 1987; Mrosovsky, 1991). Our results showed that PDF did not enhance significantly the locomotor activity, except at CT 4. Since microinjection of PDF into the optic lobe during the day increased the spontaneous activity of the brain efferent (Saifullah and Tomioka, 2003), the enhancement of activity by PDF at CT4 may be related to the increased neuronal activity. However, considering that non-consistent enhancement was induced by PDF injections at other CTs, the increased neuronal activity is not positively related with the overt locomotor activity. It seems thus likely that PDF did not cause the phase shifts of the clock through the feedback of the activity but through more direct influence on the circadian clock.

It is hypothesized that PDF is an output molecule that regulates the locomotor activity in some insects. Findings supporting this hypothesis are that PDF and PERIOD often coexist not only in *Drosophila* but also in the cricket *Teleogryllus commodus* (Helfrich-Förster, 1995; Lupien *et al.*, 2003), that flies lacking PDF often become arrhythmic in constant darkness (Renn *et al.*, 1999), and that the restoration of rhythmic locomotor activity after transection of the

optic tracts in cockroaches has a strong correlation with regeneration of PDF-immunoreactive neurons (Stengl and Homberg, 1994). Our result does not contradict the hypothesis, although the injection of PDF into the optic lobe did not cause a significant increase in activity at most CTs. Immunohistochemistry revealed two groups of PDF immunoreactive neurons in the optic lobe, one at the proximal medulla and the other near outer chiasma (Okamoto *et al*., 2001; Chuman *et al*., 2002). Some of those at the proximal medulla projected to the central brain and the rest were intrinsic in the optic lobe (Tomioka, K., unpublished observation). Thus there is a possibility that their functional role might be different in the optic lobe and in the central brain. It might play a role in phase setting of the clock in the former and in regulation of the activity in the latter. This issue should be addressed in future studies.

Comparison with cockroaches

The cricket PRC for PDF differs from that of the cockroach *L. maderae,* which shows statistically significant delays only at the late subjective day (CT8-12) and no significant advance (Fig. 6) (Petri and Stengl, 1997). The reasons for the difference may be as follows: In cockroaches, the effects of PDF might occur through mutual coupling between the two optic lobe clocks, since they are known to be tightly coupled (Page, 1981, 1985) and PDF is suggested to be involved in the coupling mechanism (Stengl and Homberg, 1994; Petri and Stengl, 1997). However, we used crickets with a single optic lobe removed so that the resultant phase shifts are attributable to the shifts of a single clock. Another possibility may be that the physiological roles of PDF are different between these two species.

It may be also noteworthy that in *L. maderae*, the authors injected cricket's (*A. domesticus*) PDF (Petri and Stengl, 1997). Although the structure of PDF has not been revealed yet in *L. maderae*, it seems reasonable to assume that it differs from that of *A. domesticus*, since the PDF of the other cockroach (*P. americana*) differs at 4th amino acid residue from that of *A. domesticus* (Rao and Riehm, 1993). It was shown that substitution of only a single residue had certain effects on relative potency of PDH in crustaceans (Rao and Riehm, 1989). Thus, in *L. maderae*, it deserves to analyze the effect of conspecific PDF on its circadian rhythm for detailed comparison with our results.

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