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Source: Zoological Science, 18(2) : 151-158

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.151

ZOOLOGICAL SCIENCE **18**: 151–158 (2001)

Ultradian Firing Rhythm of Neurosecretory Cells Producing an Insulin-related Peptide in the Silkmoth *Bombyx mori*

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ABSTRACT—There are four pairs of neurosecretory cells producing bombyxin, an insulin-related peptide, in the brain of *Bombyx mori*. In the present study, electrical signals of the bombyxin-producing cells were identified and short- and long-term activity patterns of the cells throughout the pupal period were analyzed. Activity pattern of the cells was characterized by periodic discharges of a volley of action potentials. Periodicity of the electrical activity depended on the temperature and mean±SD of periods was 34.7±4.8 min at 26°C. Four cells in a brain hemisphere were functionally heterogeneous: individual cells had specific activity amplitudes and rhythms, thereby suggesting that an ultradian activity rhythm of a cell originates from its intrinsic pacemaker activity. There was no significant diel (circadian) oscillation in the electrical activity. Daily activity patterns had a maximum at an early stage (day-2 or 3) of pupal period. Periodic secretion of bombyxin, like insulin, seems to be an efficient temporal pattern for hormonal actions.

INTRODUCTION

Insulin is a peptide hormone regulating cellular metabolism and growth of vertebrates. Secretion of insulin, like many other hormones, is characterized by a pulsatile ultradian rhythm and fluctuation of plasma insulin concentration due to the pulsatile secretion is important for maintaining hormonal actions (e.g., Matthews, *et al*., 1983; Matthews, 1991).

Insects have an insulin-related peptide hormone, bombyxin that was first identified in the head of silkmoth, *Bombyx mori* (Nagasawa *et al*., 1984, 1986). Bombyxin comprises highly heterogeneous molecular forms that are generated from complex multi-family genes (Iwami, 1995; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997, 1998). Bombyxin secretion is assigned to four pairs of neurosecretory cells with a particularly large soma in *Bombyx* (Mizoguchi *et al*., 1987) or to a large number of cells in another silkmoth *Samia cynthia cicini* (Yagi *et al*., 1995). These characteristic molecular and cellular organizations for production and secretion of bombyxin indicate that bombyxin is one of the most important peptides in insects.

Physiological functions of bombyxin are not fully understood. Bombyxin obtained from *Bombyx* elicited metamorphosis in brain-removed dormant pupae of the moth *Samia*, by acting on the prothoracic gland to stimulate the synthesis and release of ecdysteroid (Nagasawa *et al*., 1984). However, the peptide failed to activate the prothoracic gland of *Bombyx* (Ishizaki *et al*., 1983). Bombyxin injected into a feeding larva induced decreases in haemolymph trehalose and fat body glycogen, major storage carbohydrates in insects (Satake *et al*., 1997), but the peptide was ineffective for carbohydrate metabolism in the adult moth (Satake *et al*., 1999). The titer of bombyxin in the haemolymph becomes maximal during adult development (Saegusa *et al*., 1992), thereby suggesting that bombyxin is a factor regulating growth and development. It has been reported that bombyxin binds to specific receptors on ovarian cells (Fullbright *et al*., 1997) and that it induces meiosis in ovarian cells (Orikasa *et al*., 1993) or morphological changes in a cell line derived from the ovarian tissue (Tanaka *et al*., 1995).

To elucidate physiological functions of a neurosecretory cell and its products, it is vital to determine temporal patterns of it activity and mechanisms that control secretion from the cell. As noted with neurosecretory cells releasing pheromonotropic peptides (Ichikawa, 1998), monitoring electrical activities of a cell (or a set of cells) is suitable for the purpose.

Four pairs of bombyxin producing cells of *Bombyx* have a readily identifiable soma in the dorsomedial region of the brain and thick axon terminals in the corpora allata (CA) to release the hormone into the haemolymph (Ichikawa, 1991). In the present study, electrical signals of the bombyxin producing (BP) cells were detected extracellularly from the CA in pupae and pulsatile activity patterns were revealed.

MATERIALS AND METHODS

Fifth instar larvae of commercially available F1 hybrid of *Bombyx mori* (Kinshu × Showa) were purchased from a supplier and reared on an artificial diet. The larvae were placed at 26±1°C under a 16-hr light/8-hr dark photoperiod.

The ventral part of the thorax of a pupa at 6–10 hr after ecdysis was fixed with paraffin to a platform at an angle of 45°. The whole

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body except for the head of the pupa was placed in a plastic chamber and the chamber was filled with CO_2 gas for purposes of immobilization. Small pieces of head cuticle and part of the fat body in the head capsule were removed to expose the brain and the CA.

For long-term monitoring of BP cells' activities, CA was introduced into a suction electrode filled with a physiological saline containing 1% streptomycin sulfate (Sigma). A piece of silver wire serving as an indifferent electrode was made to contact the haemolymph, and the cuticular window was sealed with melted paraffin. The pupae was usually placed in an incubator kept at 26±1°C and illuminated at the same light/dark cycles, using a fluorescent lamp. Light intensity was about 100 lx. Under these experimental conditions, pupae usually shed abdominal cuticle 10 or 11 days after pupation.

For intracellular stimulation and recording, the sheath of the brain was partially removed, using a fine tungsten needle. Under visual guidance, a soma of a BP cell was penetrated with a glass microelectrode filled with 3M KCl. The microelectrode was connected to a preamplifier, which allowed simultaneous recording and current injection *via* a bridge circuit. An action potential was induced from the soma by passing a depolarizing current pulse (1–5 nA, 3 ms in duration). After all four somata of BP cells were impaled, they were removed, using a fine tungsten needle.

All electrical signals were amplified, digitized, and stored on a computer equipped with an analog/digital converter (1401plus, Cambridge Electronic Design, Cambridge). Spikes from BP cells were discriminated using spike-sorting software (Spike 2, Cambridge Electronic Design). Periods of firing activity rhythm were estimated by chi square periodogram (Sokolove and Bushell, 1978).

RESULTS

Identification of spikes of Bombyxin-producing cells

Electrical signals were recorded from the CA where BP cells have axon terminals. Because other types of neurosecretory cells such as PTTH producing cells also terminate there (Ichikawa, 1991), one should extract electrical signals (action potentials or spikes) of BP cells from multiunit neural signals. Several classes of spikes, usually discriminated based on their specific amplitudes and/or waveforms, were divided into two groups with significantly different amplitudes, large and small. To identify spikes of BP cells, each soma of a BP cell located in the dorsomedial region of a contralateral brain hemisphere was impaled and injected with current pulses. The electrical stimulation always induced large spikes regardless of the BP cell impaled (Fig. 1). When four large somata of BP cells were surgically removed, large spikes disappeared and only the small group of spikes remained (data not shown). Thus, it became evident that the large spikes originated from the BP cells.

Ultradian rhythmic activity

Spontaneous firing activities of BP cells were recorded from 40 male and 10 female pupae for 7–11 consecutive days. There were no significant differences in activity patterns

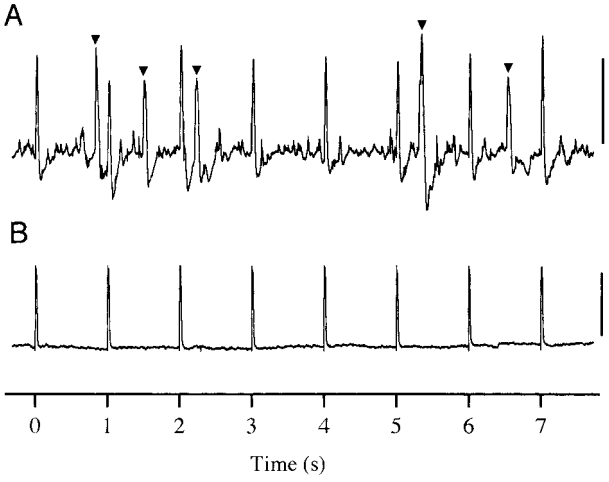

Fig. 1. Identification of spikes of a bombyxin-producing cell. Intracellular current injections into the cell at an interval of 1s induce large action potentials (B) that can be recorded as large extracellular spike potentials at the corpus allatum (A). Large spikes originating from other bombyxin-producing cells (arrowheads) are seen as well as small spikes of unknown origins. Scale bars: 0.1 mV (A), 50 mV (B)

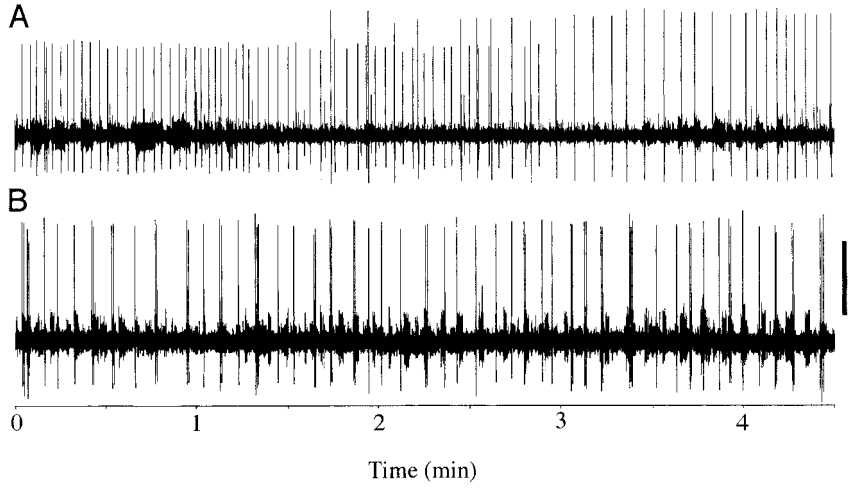

Fig. 2. Spike trains of bombyxin-producing cells. (A) One unit exhibiting relatively smaller spikes terminates firings at around middle of the record while another unit with larger spikes start to fire. (B) A cell sometimes produces doublet or triplet spikes. A and B are from different animals. Scale bar: 0.1 mV.

between sexes. Although amplitudes and waveforms of BP cells' spikes gradually changed with adult development, significantly large spikes of the cells were maintained throughout development. This feature greatly facilitated analyses of the firing activity of BP cells.

A train of spikes produced by a single BP cell could be often discriminated from those of other BP cells because of a specific amplitude and different timing of firing. A BP cell usu-

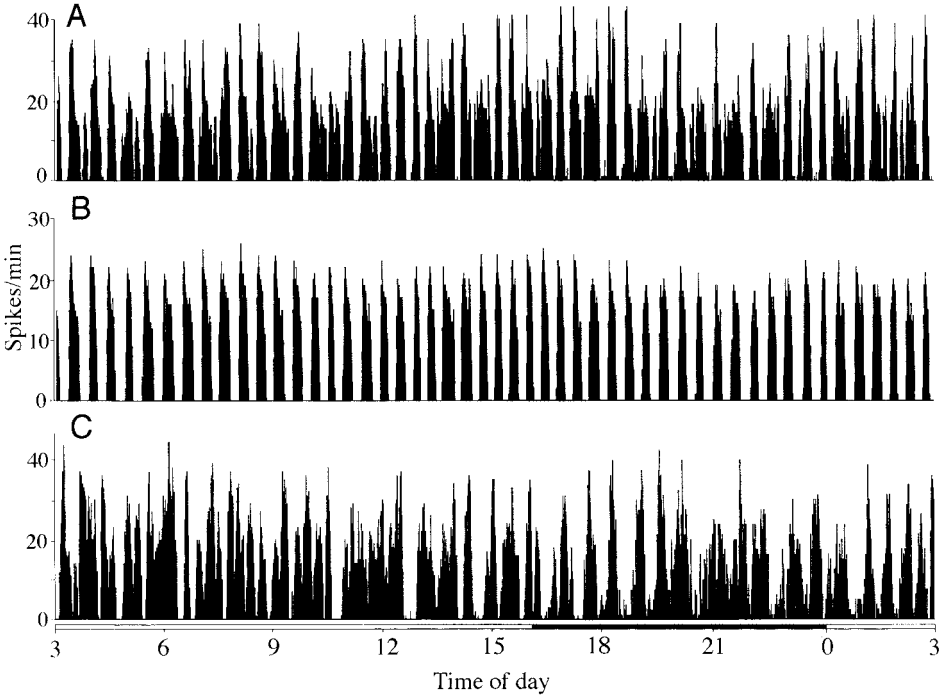

Fig. 3. Typical firing profiles of bombyxin-producing cells. (A) Multiunit activity pattern with a relatively regular rhythm. Total number of spikes (N) is 18750. (B) Pulsatile activity rhythm of a dominant single unit extracted from the record A. N=11525. Mean period is 28.8 min. (C) Multiunit activity pattern with complex rhythmicity. N=17263. Photophase and scotophase are shown by white and black bars at the bottom, respectively.

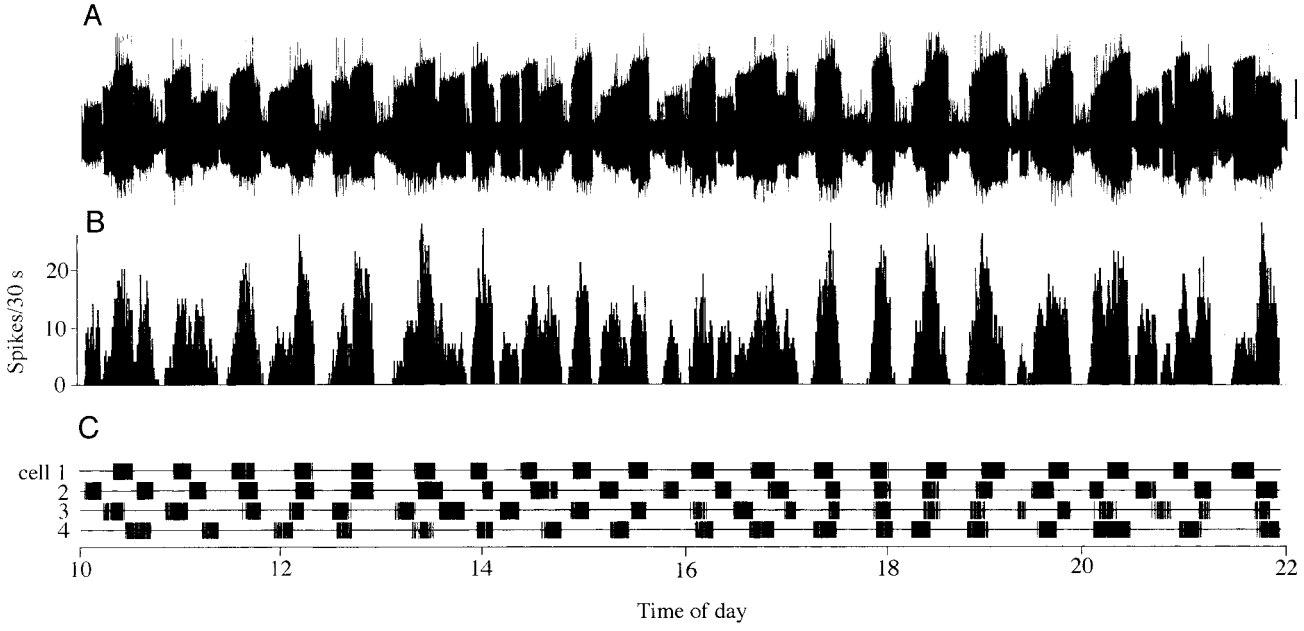

Fig. 4. Cell specific activity rhythms of individual cells. (A) Original record of electrical signals. Scale bar: 0.1 mV. (B) Complex multiunit activity rhythm extracted from the record A. Total number of spikes during the period of 12 hr is 8250. (C) Firing patterns of four cells extracted from record A. Each vertical bar represents the timing of each spike. The mean period of rhythm and the number of spikes of each cell are as follows: cell 1, 33.7 min, 3070 (37%); cell 2, 31.5 min, 2120 (26%); cell 3, 31.7min, 1492 (18%); cell 4, 39.4 min, 1565 (19%).

ally produced single spikes at a relatively constant interval of about 2 s, except at the start and end of a spike train (Fig. 2A). Some cells showed a burst of a few or several spikes generated at a short interval of about 0.5 s (Fig. 2B).

Activity pattern of a population of BP cells was characterized by rhythmic discharges of trains of spikes, though rhythmicity did vary from animal to animal. In about half the number of the pupae examined, the rhythmic pattern was relatively regular and simple (Fig. 3A), while activity patterns of others were complex and appeared to be a mixture of different rhythms (Fig. 3C). The distinct rhythmic pattern in the former type was due to a dominance of a particular single unit that accounted for about 60% of the entire population of spikes (Fig. 3B). Pulsatile activity of the dominant unit was quite regular and a maximum firing rate was usually about 20 spikes/min. There was no apparent diel change in the activity. Rhythm periods of dominant units measured on day-3 in 16 pupae varied from 26 to 48 min and mean±SD of such ultradian rhythm periods was 34.7±4.8 min at 26±1°C. Dominant single units usually accounted for 50-65% of the total activity of BP cells. Minor components of activity patterns consisted of spikes originating from one or two units with a periodicity that usually differed from that of the dominant unit.

When there were significant differences in spike amplitudes among the four units, four classes of spikes could be clearly discriminated in the record showing a complex pattern of multiunit activity (Fig. 4). Production of a train of spikes in each cell lasted for 7–20 min and such bursting activities were repeated at an interval of 25–40 min. Each cell revealed a specific periodicity and mean periods of the four cells were no longer identical. The shortest period was 31.5 min and the longest 39.4 min; the periods differing by 7.9 min. There was no stable phase-relationship between activity rhythms of any pair of cells and this situation made the apparent pattern of multiunit spikes complex.

The number of spikes produced by a single cell during a long period of time (12 hr) differed significantly: spikes of the most active cell accounted for 38% of the total number of spikes while those of the least active cell made up for only 17%. Similar cell-specific rhythms and heterogeneous activity profiles in a population of BP cells were observed in two other pupae.

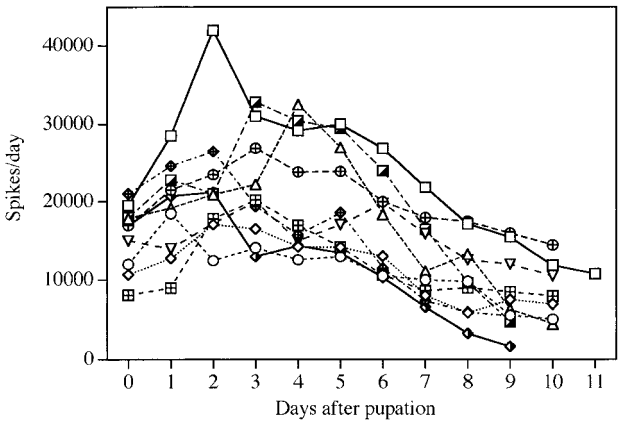

Fig. 5. Daily changes in multiunit activity of bombyxin-producing cells during the pupal period. Different symbols indicate different pupae.

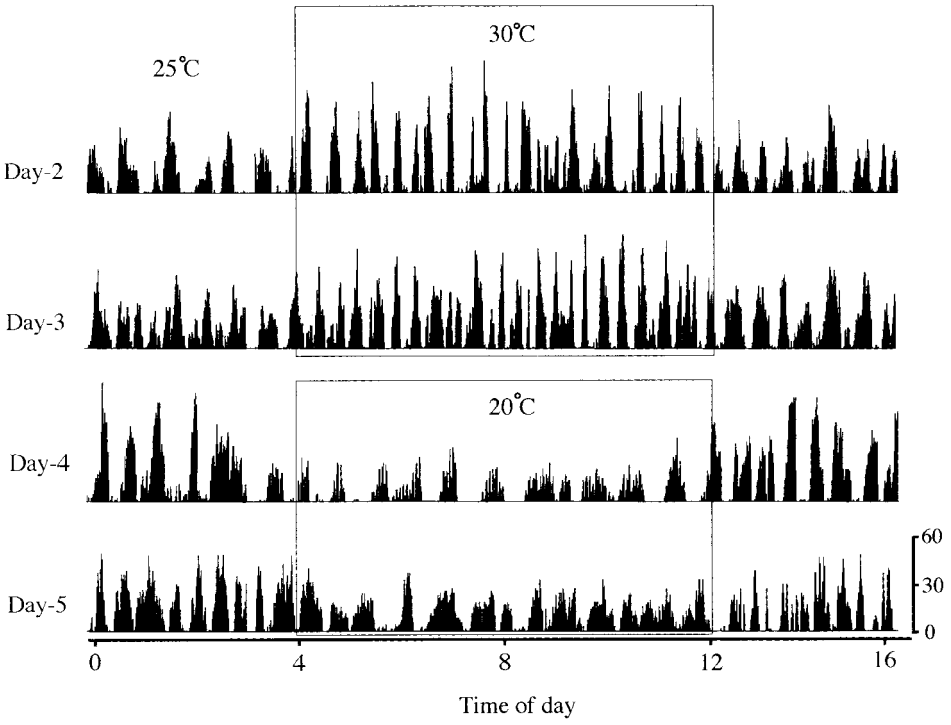

Fig. 6. Effect of temperature on the ultradian rhythm of bombyxin-producing cells. Temperature kept at 25°C was raised by 5°C for 8 hr on days-2 and 3 or lowered on days-4 and 5.

Daily activity patterns during the pupal period

Total numbers of spikes of BP cells counted daily usually became maximal at an early pupal stage, day-2 or day-3, and gradually decreased, though there was considerable variability in the spike numbers among individuals (Fig. 5).

Mean periods of the ultradian rhythms changed considerably within a range between 25 min and 55 min as pupal stages proceeded, but no common trend of changes was evident. The mean periods gradually shortened (n=2) or lengthened (n=2), while they became shortest at a middle pupal stage (n=3). A pupa maintained a relatively constant period around 30 min throughout the pupal period.

Temperature dependence of ultradian rhythm

To know a nature of the pulsatile activity of BP cells, effects of temperature on the rhythm were examined.

When the temperature was elevated from 25°C to 30°C for 8 hr during the middle photophase, the period of ultradian rhythm shortened and a maximum rate of firing increased (Fig. 6). In contrast, the period lengthened and the rate of firing decreased, when the temperature was shifted down from 25°C to 20°C (Fig. 6). Periodograms show that warming by 5°C shortened the mean period by 6 min and cooling by 5°C lengthened it by 8 min (Fig. 7). Thus, the activity rhythm of BP cells is highly sensitive to the temperature. The relationship between the temperature and the period of the ultradian rhythm is shown in Fig. 8. The regression line indicates that the rhythm becomes fast 1.83 times as the temperature is increased by 10°C. The value is the temperature coefficient (Q_{10}) and mean±SD of Q_{10} was 2.21±0.41 (n=5).

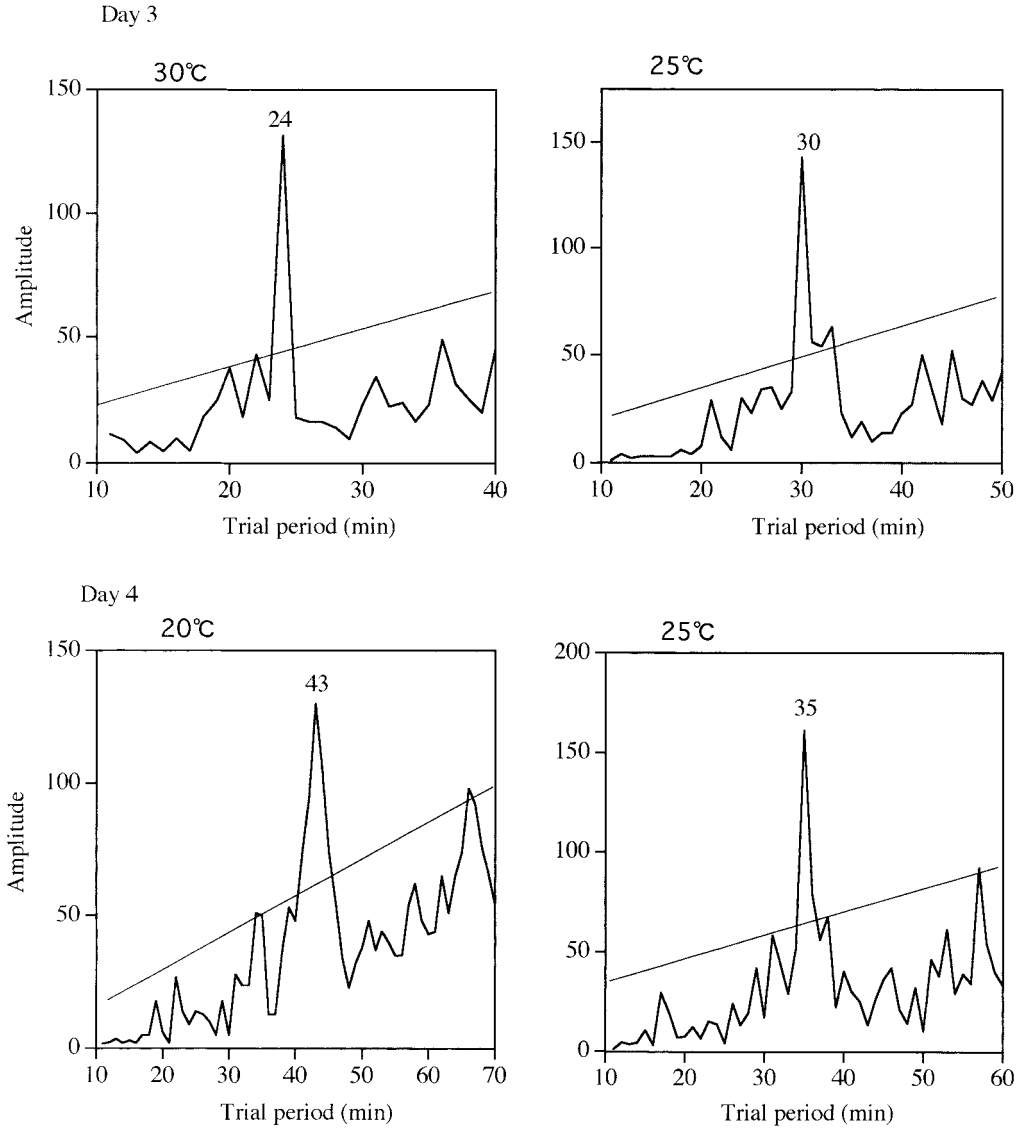

Fig. 7. Chi square periodograms of firing activity rhythms of bombyxin-producing cells under different temperatures. Each straight line indicates a 95% significance level of rhythmicity. Original records for the analyses are illustrated in Fig. 6.

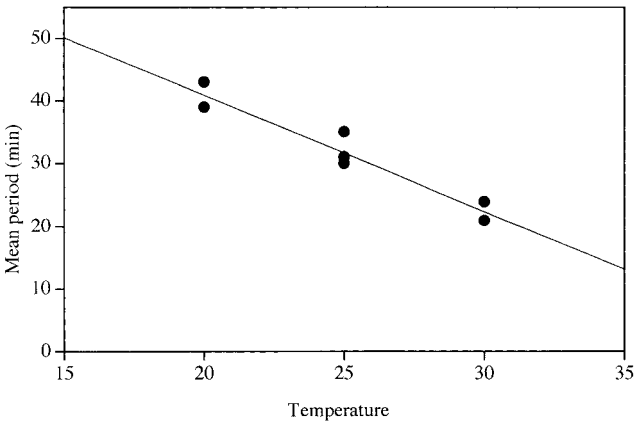

Fig. 8. Temperature dependency of the ultradian activity rhythms. Original data are illustrated in Figs. 6 and 7.

DISCUSSION

Long-term, chronic recordings of insect neurosecretory cells have been done on PBAN-producing cells in the suboesophageal ganglion of *Bombyx mori* (Ichikawa, 1998). Because the cells have an unique axonal pathway, there is no problem in identifying action potentials recorded extracellularly. In contrast, it is important to determine if a recorded action potential originated from a BP cell, because the cell sends an axon to the CA via the NCC 1+2, the common axonal pathway of cerebral neurosecretory cells. There are at least three types of neurosecretory cells terminating in the CA: BP cells, PTTH producing cells and unknown ones (Ichikawa, 1991). Because terminal branches of a BP cell are thicker and more abundant than those of cells of other types, a spike potential originating from the cell is expected to be large due to a large extracellular current flowing out from thick branches. The spike of BP cells was apparently large (Fig. 1) and this facilitated identification of spikes. Origins of small spikes were not determined. However, analysis and identification of small spikes may be important because a fraction of small spikes possibly originates from a putative clock neuron that expresses period gene and is involved in circadian rhythm in the silkmoth, *Antheraea pernyi* (Sauman and Reppert, 1996).

Each cell appears to have specific rhythmicity (Fig. 4). If electrical activities of four cells are regulated by a putative, common pacemaker (ultradian oscillator), their activity rhythms should either be synchronized or significantly phase-locked. The free running rhythm of a cell may represent its inherent oscillatory property. The period of the rhythm strongly depends on the temperature (Fig. 5) and Q_{10} is about 2.2 (Fig. 7), thereby suggesting that biochemical oscillatory mechanisms may drive the rhythmic activity of the cell. However, the result can not exclude a possible mechanism that coordinates activity rhythms of whole cells. In a preliminary experiment, simultaneous recordings from right and left corpora allata revealed a significant correlation between electrical activities of right and left groups of BP cells.

Many hormones, neuropeptides and biogenic amines in vertebrates are secreted in an ultradian pulsatile fashion (Matthews, 1991). For example, insulin secretion shows a rapid (10−14 min) and a slow oscillation (50−100 min), superimposed on circadian fluctuation. The fast oscillation appears to be an inherent feature of cellular mechanisms of secretion and the slow, circhoral oscillation is in part generated by a negative feedback loop linking glucose and insulin secretion (Polonsky *et al*., 1998). To examine feedback mechanisms involved in bombyxin secretion, a large amount of glucose (or bombyxin II) was injected into a pupa in preliminary experiments, but it induced no significant change in the activity pattern of BP cells. Glucose may be no longer effective in a pupa, as demonstrated in an adult moth, though a glucose injection into a feeding larva does induce bombyxin secretion (Satake *et al*., 1999; Masumura *et al*., 2000).

It is evident that many neuroendocrine systems rely on pulsatile patterns of secretory activity to maintain biological functions (Belchetz *et al*., 1978; Norstedt and Palmiter, 1984; Clark *et al*., 1985; Matthews, 1991; Windle *et al*., 1998). Hormone-specific periodicity of secretion seems to be important for efficient actions of the hormone. For example, circhoral (one pulse per h) stimulation of the pituitary gland with gonadotropin-releasing hormone (GnRH) is effective for a maintained gonadotropin secretion while high-frequency or continuous exposure to GnRH results in desensitization of the pituitary response (Belchetz *et al*., 1978; Wildt *et al*., 1981). Strong desensitization or down-regulation due to prolonged exposure to an agonist is a common property of many membrane receptors, including insulin receptors (Gustafson *et al*., 1999). Ultradian activity rhythm of the bombyxin-releasing cells may also be important for actions of bombyxin on target tissues. Because bombyxin II injected into a neck-ligated larva seems to be rapidly removed from the haemolymph by degradation, binding to target cells' surface receptors and subsequent incorporation into the cells (Satake *et al*., 1997), a similar rapid removal of bombyxin secreted into the pupal haemolymph may also occur. Since a half-life period of bombyxin in the haemolymph is less than 1 hr during an initial phase of removal, pulsatile secretion of bombyxin at an interval of 30−40 min may be significant to fluctuate the hormone titer in the haemolymph. A putative bombyxin receptor has been found on ovarian cells of *Bombyx mori* and two other species of moth and it has structural similarities to the mammalian insulin receptor (Fullbright *et al*., 1997).

Secretion of an insect peptide hormone is usually assigned to multiple, clustered neurosecretory cells. For example, five pairs of cells produce a neuropeptide for activating sex pheromone production in *Bombyx mori* (Ichikawa *et al*., 1995, 1996), and 16 pairs of cells are involved in the production of a bombyxin-immunoreactive material in *Samia cynthia ricini* (Yagi, *et al*., 1995). One important question is whether all those cells contribute equally to secretion of the hormone. The present study revealed that four pairs of cells are electrophysiologically heterogeneous. The amount of hormone released from a cell may be practically in proportion to the electrical activity of the cell because there is a close simi-

larity between changes in electrical activity (Fig. 5) and in the titer of bombyxin immunoreactive material in the haemolymph during adult development (Saegusa *et al*., 1992). Thus, they do not equally contribute to the secretion of bombyxin at least during the pupal stage. One or two dominant cells are significantly more active than others: the former produces >50% of the entire number of action potentials (Figs. 3 and 4). Mizoguchi *et al*. (1990) reported stage-specific, unequal distribution of bombyxin-immunoreactive materials among the four cells in a hemisphere of the larval *Bombyx* brain: two are stained more intensely than were the other two cells, thereby suggesting different secretory activities of those cells. Similar heterogeneous distributions of firing activities were observed among five neurosecretory cells releasing PBAN in a female moth of *Bombyx* (T. Ichikawa, unpublished observation). If all bombyxin-producing cells become equally active as the dominant one, they can secret at least a 2- or 3-fold larger amount of hormone. Such an active state may be evident in an adult male 3–6 hr after eclosion, at which the bombyxin titer transiently increases to 5-fold larger than the titer at the pupal stage (Satake *et al*., 1999).

ACKNOWLEDGEMENTS

I thank M. Ohara for language assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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(Received October 10, 2000 / Accepted November 3, 2000)