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Physiology

Activity patterns of neurosecretory cells releasing pheromonotropic neuropeptides in the moth *Bombyx mori*

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ABSTRACT **Short- and long-term firing patterns of neurosecretory cells releasing pheromonotropic neuropeptides in the silkworm moth *Bombyx mori* were examined. The cells showed three types of rhythmic changes in firing activity. Bursting activities with an interval of several seconds were synchronized with rhythmic abdominal motions for calling behavior. A slow fluctuation in firing activity over a period of several minutes depended on cyclic alternations of the flow of hemolymph. The electrical activity displayed a diel rhythm that related to light/dark cycles of the environment and sex pheromone titers in the pheromone gland. In addition to a transient inhibition of firing caused by a tactile or light stimulus, a long-term permanent inhibition was induced by mating with a fertile male. Thus, the insect neurosecretory system is highly coordinated with physiology and behavior in *Bombyx mori* and is influenced by external stimuli.**

Female moths extrude the pheromone gland and emit sex pheromone to attract males. The calling behavior and sex pheromone production exhibit a diel periodicity with peak pheromone titers occurring during the photophase or scotophase. This diel periodicity of sex pheromone production in many species of moths is under the control of pheromonotropic neuropeptides, such as a pheromone biosynthesis-activating neuropeptide (PBAN) and PBAN-like factors (1, 2). PBANs have been identified from three species of moths (3–5). Analyses of cDNAs encoding PBANs in *Bombyx mori* and *Helicoverpa zea* showed that the neuropeptide is generated along with four additional family neuropeptides from a common precursor polyprotein that is translated from a single mRNA (6–8). In *B. mori*, the four neuropeptides, including a hormone inducing embryonic diapause, share a conserved pentapeptide amide at the C-terminal and have substantial pheromonotropic activity (8). Six pairs of somata of neurosecretory cells expressing the gene for the precursor protein are aggregated into three clusters localized at the ventral surface of the anterior, medial, and posterior neuromeres of the suboesophageal ganglion (SOG) (9). Surgical ablation of the anterior and medial clusters of somata at an early pupal stage greatly impaired pheromone production at the adult stage, whereas the same operation on the posterior cluster impaired diapause induction rather than pheromone production, thereby suggesting functional specialization of the three classes of neurosecretory cells (10). Intracellular dye injection revealed complete structures of individual neurosecretory cells, including a unique axonal pathway and neurohemal terminals (11). Five axons from two anterior and three medial cells project to the corpus cardiacum (CC) after passing through a branch of the maxillary nerve, nervus corporis cardiaci ventralis (NCC-V), that runs beneath the cuticle of the head. Many varicose terminal branches in the CC and associated nerves of the CC indicate that pheromonotropic neuropeptides synthesized in the somata are transferred to neurohemal areas and are then released into the hemolymph to reach the pheromone gland (11).

To elucidate physiological functions of these neurosecretory cells and their products, it is vital to determine temporal patterns of their activity and mechanisms that control secretion from the cells. Because an electrical action potential of a neurosecretory cell triggers secretion related mechanisms, electrical signals from the cell are most suitable to monitor secretory activity in real time and over a long period of time. In the present study, I made long-term chronic recordings from neurosecretory cells releasing PBAN and its related peptides of *B. mori* and characterized their complex firing activities that are closely related to calling behavior, circulation of hemolymph, and (diel) changes in pheromone titers in virgin and mated females.

MATERIALS AND METHODS

Insect. Commercially available F_1 hybrids of *B. mori* (Tokai × Fuyo or Shunrei × Shogetu) were used. Pupae and adult moths were placed at 26 ± 1°C under a 16-h light/8-h dark photoperiod. Some females were mated with a intact (fertile) male or a sterile male. The latter was a male whose testes had been surgically removed at an earliest pupal stage or a male whose penis was excised before mating. The latter, like an intact male, can hold a female with its claspers but cannot ejaculate semen.

Electrophysiology. After removing all legs of a 1- to 2-day-old female, the ventral part of the thorax and wings were fixed to a platform at an angle of 60° with paraffin. The platform had a stage on which a male could approach the female for mating. The female struggled to get free from restraint for a few minutes or a few hours. After the calling behavior started, the head was immobilized with paraffin and part of the cuticle over the SOG was removed to expose the NCC-V. To eliminate contamination of signals from cerebral neurosecretory cells that project axon collaterals into the NCC-V via the CC (12), the nerve was cut and its proximal stump was introduced into a suction electrode. A piece of silver wire serving as an indifferent electrode was made to contact the hemolymph, and the cuticular window was sealed with vaseline. To monitor abdominal movement, the fourth or fifth abdominal segment of the female was placed between a pair of infrared light emitting diodes and a phototransistor. Output of the phototransistor was practically linear within a dynamic range of 5 mm. To record electrocardiograms, a piece of Teflon-coated silver wire was placed near the dorsal loop of the aorta and another one was inserted into the anterior portion of the thorax. Wounds were sealed with a quick-drying glue. All electrical signals were amplified, digitized, and stored on a

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Abbreviations: PBAN, pheromone biosynthesis-activating neuropeptide; SOG, suboesophageal ganglion; CC, corpus cardiacum; NCC-V, nervus corporis cardiaci ventralis.

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computer equipped with an analog/digital converter [MacLab 8s (AD Instruments, Castle Hill, Australia) or 1401plus (Cambridge Electric Design, Cambridge)]. Because an action potential (spike) of a neurosecretory cell is much slower than that of an ordinary neuron (12), signals from the neurosecretory cells were usually filtered at 30–100 Hz and digitized at 400–1,000 Hz. Spike-sorting software (SPIKE 2, Cambridge Electric Design) failed to discriminate the second action potential of two close action potentials. Failures of discrimination by visual inspection were at most 10% and were corrected in Fig. 1, but underestimates of rates of action potentials in other figures were not corrected. During chronic recordings, the animal was illuminated at the same light/dark cycles, using a fluorescent lamp. Light intensity was about 150 luxes. For stimulation, the lamp was manually turned on or off. For a tactile stimulus, a round tip of a pencil was pushed against a tip or a lateral part of the abdomen. Cervical connectives were transected by using microscissors after removing a part of the cuticle above the connectives.

HPLC Analysis of Bombykol Titer. The sex pheromone glands of a female were excised and placed in 500 μl of hexane for 10 min. The extracts were injected onto an HPLC column (Nucleosil $5NO_2$, Chemco Pak, Osaka, Japan) that was installed in an HPLC system (Hitachi L-6200). Chromatographic conditions were the same as described by Arima *et al.* (13). Titer of pheromone (Bombykol) was monitored with absorbance at 230 nm.

RESULTS

Electrical action potentials of the neurosecretory cells releasing pheromonotropic peptides were recorded extracellularly from a proximal stump of the NCC-V for 3–5 consecutive days (n = 47). Fig. 1 shows spontaneous bursting activities of multiple units observed in a virgin female during midphotophase. A cluster of action potentials usually appears to consist of five classes of discrete potentials with different amplitudes, as indicated in Fig. 1*A*. A preliminary experiment revealed the following characteristics of the action potentials: (*i*) average amplitudes of five classes of potentials often showed ratios of 1:2:3:4:5, (*ii*) larger action potentials usually had one or more notches indicating superposition of multiple spikes, and (*iii*) individual action potentials recorded from right NCC-V were almost completely synchronized with counterparts recorded from left NCC-V. Thus, the smallest action potential is regarded as a spike of a single cell and a larger potential is a compound action potential originating from multiple units that fire in synchrony. A class of action potentials usually occupies 13–27% total number of action potentials recorded during midphotophase. To determine the firing rates of five neurosecretory cells, average numbers of units firing in synchrony were calculated from the distribution of five classes of action potentials and were 2.53–3.58 (mean ± SD = 2.93 ± 0.24; n = 18). The rhythmic bursts of action potentials usually had a rate of 8–15 min^{-1}, which appeared to be close to the rate of periodic movement of the abdomen for calling, in which the insect contracted the lateral and dorsal musculatures of the body wall and lifted the abdomen to extrude the pheromone glands into the air. With a pair of infrared light emitting diodes and a phototransistor, I monitored the elevation of the abdomen to search for correlation with electrical activities of the cells. There is a close temporal correlation between the two events: the cells fire strongly at the rising phase of each motion of the abdomen (Fig. 1 *A* and *B*). A strong and clustered firing activity was generally accompanied by a large motion, and weak or sporadic firing activity was seen at a small motion or at an inflection of a motion. Statistical analysis of the relationship between the number of spikes and amplitude of motion gave correlation coefficients of 0.554–0.799 (mean ± SD = 0.680 ± 0.095, n = 10; Fig. 1*C*).

There was a considerable variation in patterns of abdominal movement among individuals: some showed almost regular oscillatory movement without a rest even for hours, whereas others showed intermittent oscillation. The irregularity of the

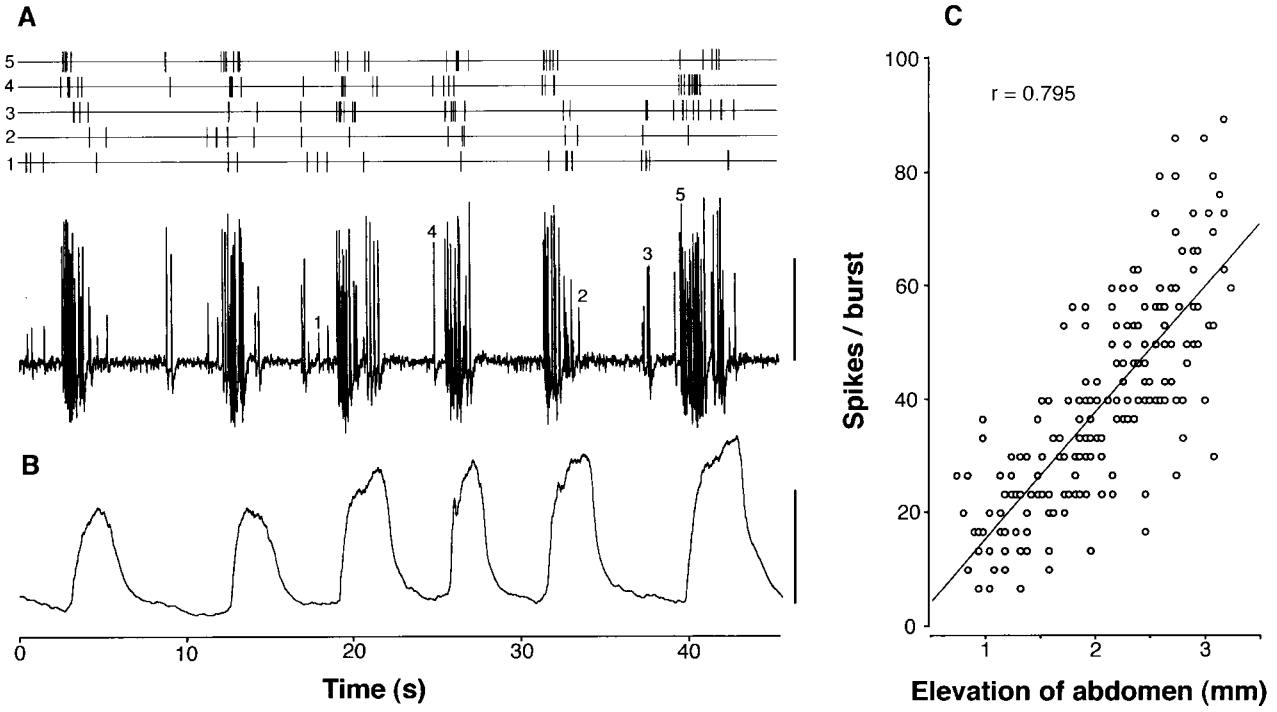

FIG. 1. Synchronization of bursting activities of neurosecretory cells with calling behavior of a virgin female of *B. mori*. (*A*) Firing of the cells. Five classes of action potentials are numbered 1–5 and timing of each potential is shown by a vertical line above the waveform trace. (*B*) Abdominal movement. Time 0 = 13 h:30 min:21 s. (*C*) Relationship between the firing activities of neurosecretory cells and the amplitude of abdominal movement. Number of spikes consisting of each burst was plotted against maximal position of the abdomen at the motion corresponding to the burst. The regression line represents $y = 22.177x - 6.859$ (n = 205). r, Correlation coefficient. [Calibration bars = 0.2 mV (*A*) and 2 mm (*B*).]

oscillatory movement usually increased with age of the moths. Firing rates of the neurosecretory cells often fluctuated slowly in accordance with change in the average position of the abdomen (Fig. 2 *A–C*). A remarkable attenuation or a brief pause of firing and movement occurred at an interval of 2–5 min. Because adult Lepidoptera show alternations in the flow of hemolymph due to a rhythmic heartbeat reversal with a similar range of intervals (14), electrocardiograms were recorded from a thoracic portion of the dorsal vessel (aorta). A higher pulse frequency (tachycardia) characterizes the period of forward beating during which the hemolymph flows from the rear to the head and backward beating with a slower pulse frequency, often started after a brief heartbeat pause (Fig. 2*D*). The average period of the heartbeat reversal rhythm, measured around a midpoint of the photophase, was 188 ± 22 s ($n = 15$). It became apparent that attenuation of the abdominal movement occurred during the periods of forward beating and early phase of the backward beating. Similar attenuation of movement was sometimes seen during the middle or later phase of the backward beating period. Because such attenuation also accompanied a reduction of firing rates of the cells, firing level of the cells appeared to depend on the average position of the abdomen rather than the heartbeat reversal itself. When waveforms of the abdominal movement and spike rates of the neurosecretory cells during different cycles of forward/backward beating were averaged after setting time of the first forward pulses to time 0, there was a close relationship between them (Fig. 3). Because the first forward pulse usually occurs at a lowered position of the abdomen, there is a steep valley in the averaged position of the abdomen.

Long-term chronic recordings revealed a diel change in firing activities of neurosecretory cells. The activities of cells in a virgin female fluctuated in synchrony with the light/dark cycles (Fig. 4). Two components were seen in the fluctuation pattern, the main one was alternation of active and inactive

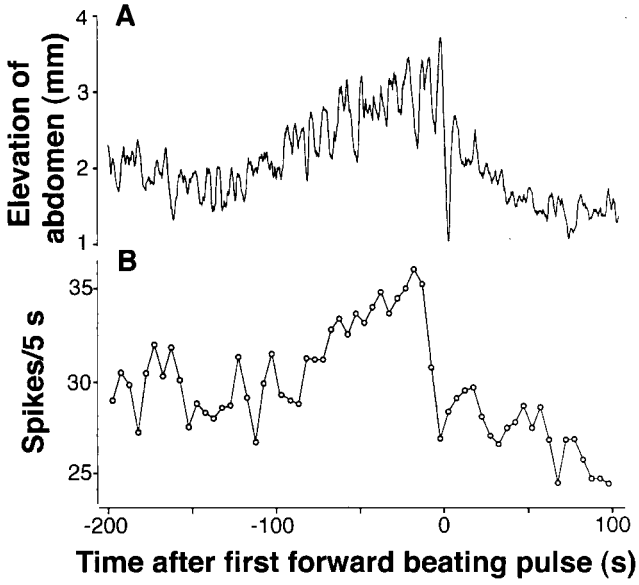

FIG. 3. Relationship between the average position of the abdomen and average firing activity of neurosecretory cells. Waveforms of the abdominal movement (*A*) and rates of spikes (*B*) during 50 cycles of forward/backward beating were averaged after setting time of the first forward pulse of each cycle (see Fig. 2*D*) to time 0.

states that had durations of about 10 and 14 h, respectively. Firing rates of the cells increased rapidly about 1 or 2 h after the onset of light and an increased level of firing activity (usually 400–600 spikes per min) continued 8–10 h before a rapid decline in the rates to near zero. The averaged duration of the active state determined at half-maximal activity was 8.9 ± 0.6 h ($n = 22$). The second, minor, component was a

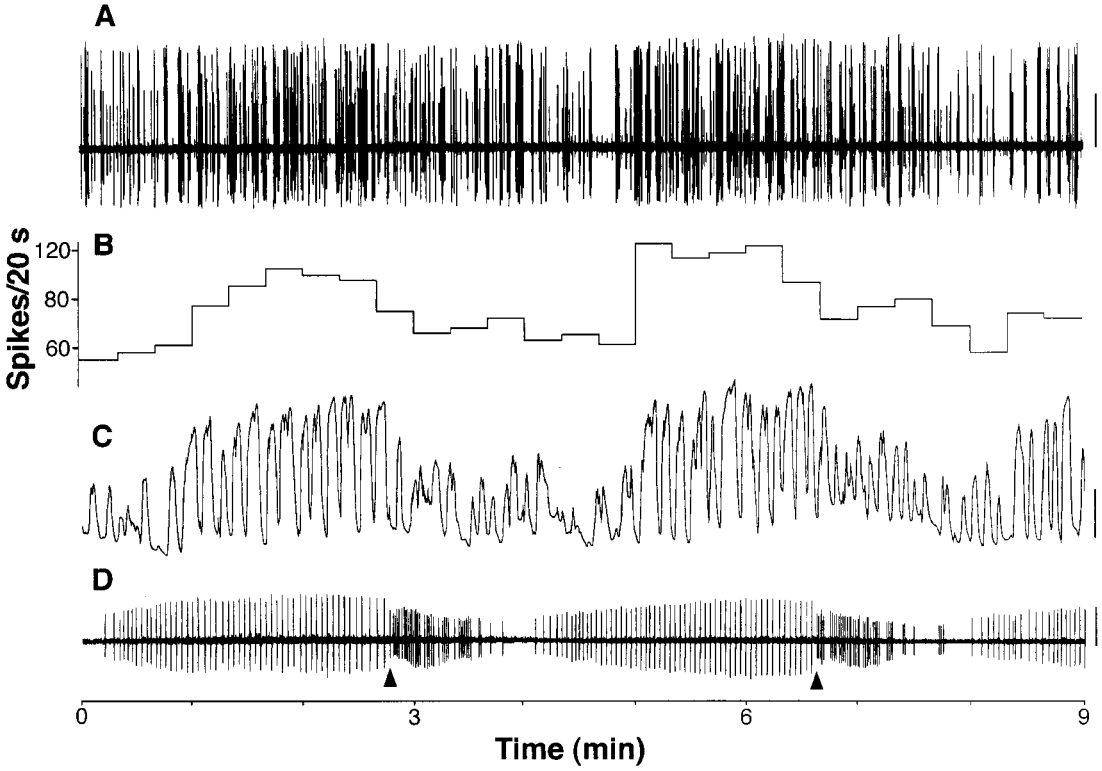

FIG. 2. Coordination of firing activities of the cells with abdominal movements and heartbeat reversals, obtained from a 3-day-old female. (*A*) Firing of the cells. (*B*) Numbers of spikes counted every 20 s. (*C*) Movement of the abdomen. (*D*) Electrocardiogram. High-frequency pulses are evident during a forward beating period and low-frequency pulses are evident during a backward beating period. The first forward beating pulse is indicated by an arrowhead. Time 0 = 14 h:21 min:43 s. [Calibration bars = 0.2 mV (*A* and *D*) and 2 mm (*C*).]

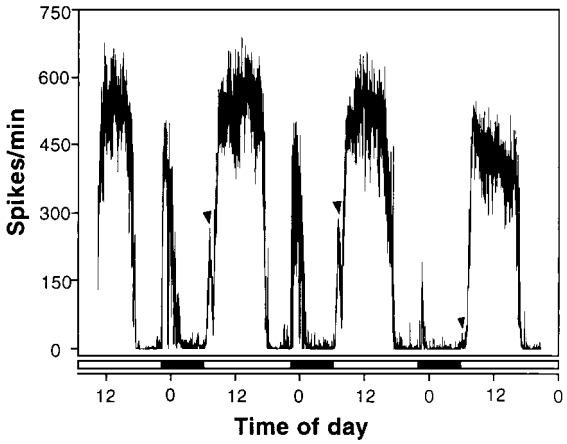

FIG. 4. Diel changes in electrical activities of neurosecretory cells in a virgin female. Numbers of spikes per minute are plotted as a function of the time of day (in hours). Transient increases in activities caused by beginning (arrowheads) or end of light are superimposed on a diel change in the activities. Light regimes (16-h light/8-h dark) are indicated by open (light) and solid (dark) bars.

transient increase in activity in response to a transition from light to dark or *vice versa*. A marked increase in firing activity began about several minutes after initiation or termination of the light. The amplitude and duration of the transient increase induced by termination of the light were usually larger and longer than these events induced by initiation of the light, although with animal to animal variations. Prolonged recordings often diminished the transient responses (Fig. 4). In a few animals, cells lacked such a response to light, even at an early period of recording.

Neurosecretory cells are most sensitive to abrupt sensory stimulation. The cells stopped firing briefly in response to light adjustments. The stimulus usually elicited a withdrawal of the tip of the abdomen and pheromone glands. Inhibition of firing of the cells did not result from an afferent signal induced by withdrawal of the abdomen, because similar responses were obtained from the same animal after transection of the ventral nerve cord between the SOG and thoracic ganglions (Fig. 5*A*). Repetitive stimulation usually induced significant decreases in amplitude and duration of the response (habituation). A similar inhibitory response of the cells and withdrawal of the abdomen were observed in response to vibration of the floor or a tactile stimulus applied to the abdomen.

Mating inactivated the neurosecretory cells. In 11 of 12 females, firing of the neurosecretory cells ceased as soon as a male touched the tip of the abdomen of the female (Fig. 5*B*) and the inactive state of the cells continued during copulation. In the exceptional one, firing was partially inhibited during copulation. With artificial interruption of copulation several minutes after the start of copulation, the cells resumed firing, but when copulation lasted for more than 40 min, usually the cells did not fire thereafter, if the mate was intact (Fig. 5*C*). For copulation with a sterile male, firing of the cells ceased during copulation but not afterward. The neurosecretory cells usually began to fire the next morning, like those in a virgin female (data not shown).

Fig. 6 shows diel changes in mean pheromone contents in virgin and mated females. As expected from firing activities of the neurosecretory cells, pheromone contents in virgin females are maximal at about midpoint of the photophase and minimal at the end of scotophase, and there is a secondary peak at the early scotophase. Mating with a intact male decreased the pheromone content exponentially and an inactive state of pheromone production continued after mating.

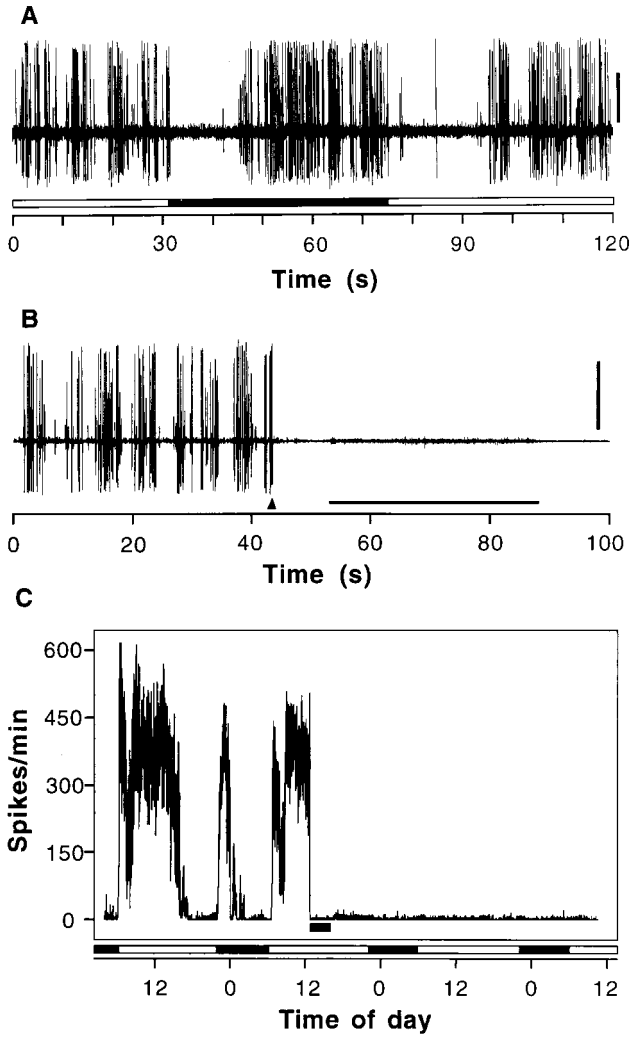

FIG. 5. (*A*) Rhythmic firing of neurosecretory cells and inhibitory responses to onset and offset of illumination recorded from a virgin female with a transected ventral nerve cord. Light and dark periods are indicated by open and solid bars, respectively. Time 0 = 12 h:7 min:8 s. (*B*) Inactivation of neurosecretory cells by mating. The cells stop firing when an active male touches the tip of abdomen of a female (arrowhead). Small noises are due to vibration produced by flapping of a male at the initial phase of copulation (horizontal bar). (*C*) Firing pattern of the same neurosecretory cells illustrated in *B* before and after a 3-h mating (horizontal bar). Light regimes (16-h light/8-h dark) are indicated by open (light) and solid (dark) bars. [Calibration bars = 0.2 mV (*A* and *B*).]

DISCUSSION

The evidence obtained shows that the neurosecretory mechanism is susceptible to sensory and environmental factors and is highly coordinated with physiology and behavior of *B. mori*. The rhythmic bursting patterns of the cells may be basically determined by the intrinsic nature of a putative central pattern generator (oscillator) in the brain–SOG complex, because isolation of the complex from the ventral nervous system did not impair rhythm generation of the cells (Fig. 5*A*). Because the brain–SOG complex is implicated in calling behavior in *Manduca sexta* (15), the oscillator for the neurosecretory cells may also control motor neurons involved in the abdominal movement for calling behavior or may be strongly coupled to another oscillator for the behavior. As noted in vasopressin neurons in the mammalian neurohypophysis (16), rhythmic bursting of neurosecretory cells may facilitate release per spike and avoid secretory fatigue at their terminals by imposition of silent periods.

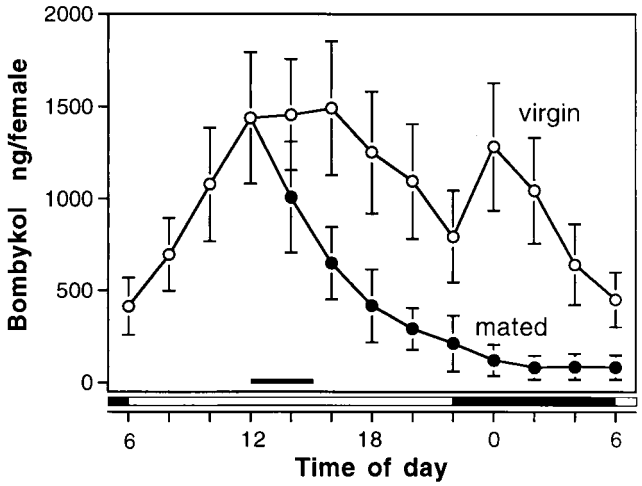

FIG. 6. Diel changes in Bombykol contents in virgin and mated females. One-day-old females were kept virgin or were mated with a fertile male for 3 h (horizontal bars) before artificial separation. Data are the mean ± SD (n = 10). Light regimes (16-h light/8-h dark) are indicated by open (light) and solid (dark) bars.

Because the neurosecretory cells release peptides into the hemolymph at the neurohemal site in the head, higher electrical (secretory) activities during a backward flow of hemolymph would seem reasonable for a rapid delivery of the released peptides to target organs in the abdomen. The rapid delivery may minimize degradation of the hormone by peptidase(s) present in hemolymph (17). In a preliminary experiment, an injection of a dye (methylene blue) into the heart during a backward beating period revealed that hemolymph mainly flowed out from the rear end of the heart in the vicinity of the pheromone gland. Thus, the gland may be periodically exposed to hemolymph containing newly released concentrated neuropeptides. Periodic or intermittent stimulation of the pheromone gland with concentrated neurohormones is likely to be more effective for activation of pheromone production than continuous stimulation with diluted ones, because the latter may induce desensitization of a putative receptor for the neuropeptides. Such desensitization due to prolonged exposure to an agonist is a common property of many G protein-coupled receptors (18). A kind of G protein-coupled receptor appears to mediate pheromonotropic action of PBAN (19).

Periodic heartbeat reversals may indirectly affect firing activities of neurosecretory cells by inducing changes in volume of the hemolymph in the abdomen, which in turn determines the blood pressure and basal position of the abdomen. The abdomen filled with a large amount of hemolymph may be able to perform a large complete motion. Inhibitory responses to tactile stimuli applied to the abdomen suggest that some segmental proprioceptors (20) are involved in monitoring stretch or posture of the abdomen.

Pheromone contents in the gland of virgin females show a diel fluctuation pattern (Fig. 6) that is qualitatively in accord with expectations based on diel change in the firing (secretory) activity of neurosecretory cells (Fig. 4) and the time course of a pheromonostatic mechanism after cessation of the hormone secretion (Fig. 6). This observation supports the notion that pheromonotropic neuropeptides function as circulating neurohormones, which is a generally accepted idea (1, 2). However, hormonal regulation of the sex-pheromone gland of *B. mori* does not preclude neural regulation of pheromone production. The latter mechanism is inferred from the presence of PBAN-immunoreactive interneurons that descend the ventral nerve cord and terminate in the terminal abdominal ganglion of several species of moths (21–25). Several lines of evidence suggest efferent neural regulation of the pheromone gland in *Lymantria disper* (26), and Christensen *et al.* (27, 28) proposed a neural mechanism mediated by octopaminergic neurons innervating the pheromone gland in *Helicoverpa zea* and *Helicoverpa virescens*. However, there are conflicting results concerning pheromonotropic action of the octopamine, even in *H. zea* (29, 30). Because an efferent neural mechanism is important for calling behavior and for pheromone release (31, 32), a similar mechanism may be involved in the modulation of pheromone production, in some species of moths. The close correlation between neurosecretion and calling behavior revealed in the present study suggests that the neural mechanism may be closely coupled with neural mechanisms that control neurosecretion.

In many species of moths, suppression of pheromone production after mating seems to be induced by a neural signal that originates from the abdomen and runs up the ventral nerve cord to inhibit the release of pheromonotropic neuropeptides, because transection of the ventral nerve cord failed to induce the inactivation of pheromone production after mating (33–37). The present study demonstrated that the neurosecretory cells releasing the neuropeptides were most sensitive to mechanical stimulation and their firing activities were completely suppressed as soon as mating began (Fig. 5*B*). Thus, a mechanical signal induced by physical stimulation during copulation can trigger an inactivation mechanism of the neurosecretory cells. Because mating with a sterile male failed to induce permanent suppression of the cells, a temporal inhibition caused by mechanical stimuli may be released if they cannot receive another signal that may be induced by the sperm and/or testicular factors transferred from a fertile male. A similar two-step inactivation mechanism of sex pheromone production has been inferred from observations of mated gypsy moth (33). Although a pheromone suppressive male factor in *H. zea* is apparently a polypeptide (38), identification and mode of action of a putative pheromonostatic male factor in *B. mori* remain to be determined.

Electrophysiological characterizations of the activity of insect neurosecretory cells have been restricted to cells involved in episodic events, such as ecdysis or ecdysis-related events (39–41). Numerous neurosecretory cells are anatomically mapped in the brain, but little success has been achieved in analyzing long-term activity of any particular unit because they send an axon to a common pathway (42). The SOG contains a few groups of neurosecretory cells having a unique axonal pathway beneath the cuticle, including PBAN-immunoreactive cells (11, 21–25). This situation facilitates identification of cells and long-term recordings without serious damage due to massive dissection of the head (43). The observations made in the present study may provide insight into the nature and control mechanisms of neurosecretory cell systems regulating long-term or continuous processes concerning homeostasis, metabolism, metamorphosis, and diapause (44–46).

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