Coordination Between the Electrical Activity of Developing Indirect Flight Muscles and the Firing Activity of a Population of Neurosecretory Cells in the Silkmoth, *Bombyx mori*

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The developing indirect flight muscles of pharate moths are characterized by a rhythmic discharge of a long bout of flight-pattern-like muscle potentials in the absence of contractions. The electrical activity of the dorsal longitudinal flight muscles (DLMs) in the silkmoth, *Bombyx mori*, was discernible as a cluster of many series of muscle potentials that last for several minutes on day 4 of the pupal period. The duration of the active phases and the period of rhythmic activity gradually increased to a peak value on day 7 or 8 and then declined until the end of the pupal period. Mean duration of the active phases (±SD) and the mean period of the rhythmic activity (±SD) at the peak were 38.7±6.7 min and 74.5±7.3 min, respectively. The rhythmic electrical activity of immature DLMs was closely coordinated with the rhythmic (bursting) activity of a population of neurosecretory cells that are known to produce pheromone-biosynthesis activating neuropeptide (PBAN) and its related peptides, which belong to the multifunctional peptide family, pyrokinin/PBAN. The DLMs always became active a few minutes after the neurosecretory cells, and the timing of onset of these two activities appeared to be strictly regulated by a neural mechanism. The implication of the coordinated activity for development and maturation of imaginal tissues, including the flight motor system, and possible functions of the neuropeptides in this development are discussed.

Key words: pupa, flight muscle, postembryonic development, neurosecretory cell, PBAN, ultradian rhythm

INTRODUCTION

The neuromuscular systems of holometabolous insects are extensively reorganized during pupal-adult development. Many larval muscles die or partially degenerate after the onset of metamorphosis; subsequently, functional adult muscles differentiate and grow (Hegstrom and Truman, 1996). The flight muscles in moths develop from an anlage comprising both remnants of specific larval muscles and imaginal myoblasts (Duch et al., 2000). Myoblasts migrate to template fibers, proliferate, and fuse the larval template fibers during the early and middle pupal period. Close interactions exist between motoneurons and the anlage during the early stage of muscle formation, and neural influences are essential for the proliferation of myoblasts and subsequent muscle development (Bayline et al., 2001). During the middle and late pupal period, developing flight muscles are characterized by a rhythmic discharge of flight-pattern-like electrical activity in the absence of muscle contractions (Kammer and Kinnamon, 1979); however, the functional significance of the observed rhythmic activity on the flight motor system was largely unknown.

Rhythmic firing activity was observed in insect neurosecretory cell systems in the silkmoth, *Bombyx mori* (Ichikawa, 1998, 2001a,b, 2003; Ichikawa and Kamimoto, 2003b), and the rhythm of the activity was usually coordinated with periodic alternations in the direction of peristaltic movement of the dorsal vessel (heart) (Ichikawa, 1998; Ichikawa and Okada, 2002; Ichikawa and Kamimoto, 2003). While studying the relationship between the firing pattern of neurosecretory cells that produce the pheromone biosynthesis-activating neuropeptide (PBAN) and cardiac activity, electrocardiograms of the silkmoth that were obtained at the late pupal period were often contaminated with oscillatory potentials, which were closely correlated with the rhythm of firing activity of the neurosecretory cells (Ichikawa and Kamimoto, 2003a); however, the origin of these potentials was unknown.

In this paper, we report that the oscillatory potentials, the origins of which were once unknown, originate from the developing indirect flight muscles. The close relationship observed between flight muscle activity and the firing activity of neurosecretory cells gave rise to the hypothesis that the development and maturation of the flight motor system of holometabolous insects could be regulated by neural and neurohormonal mechanisms.

MATERIALS AND METHODS

A commercially available F1 hybrid of *Bombyx mori* (Kinshu ×...
Showa) was used. In order to obtain continuous electromyogram (EMG) recordings of developing flight muscles, a piece of silver wire (0.2 mm in diameter) was inserted into the dorsal region of the thorax to make contact with the left bundles of the dorsal longitudinal muscles (DLMs) in a female pupa at 3–4 days after pupation. In some pupae, another piece of silver wire was inserted into an appropriate region of the thorax to make contact with the right bundles of the DLMs or the left bundles of the dorso-ventral muscles (DVM). A piece of silver wire that served as an indifferent electrode was inserted into the first abdominal segment. After the wounds were sealed with quick-drying glue and melted paraffin, the dorsal region of the thorax was fixed to a platform.

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**Fig. 1.** Electrical activity patterns of the developing dorsal longitudinal muscles (DLMs) at three different stages of the pupal period. A part of the cluster of muscle potentials is shown above each trace on an expanded time scale. The arrowheads point to the major active phases of muscles on day 5, because large, slow potentials of unknown origin often dominate muscle potentials. All saturated deflections of potentials in all records correspond to the slow potentials. The inset shows such slow potentials (arrows), on which tiny muscle potentials are superimposed. Scale bars: 0.2 mV.
To record the firing activity of PBAN-producing neurosecretory cells, a part of the cuticle over the subesophageal ganglion (SG) of the pupa was removed in order to expose the maxillary nerve that extends from the SG. The maxillary nerve was cut, and the proximal stump of the nerve was introduced into a suction electrode filled with physiological saline containing 0.1% streptomycin sulfate (Sigma, St. Louis, MO). A piece of silver wire serving as an indifferent electrode was placed in contact with the hemolymph, and the cuticular window was sealed with melted paraffin. All pupae were maintained at 28°C±1°C under a 14-h light/10-h dark photoperiod.

Electrical signals were amplified, digitized, and stored on a computer equipped with an analog/digital converter (1401 plus; Cambridge Electronic Design, Cambridge, UK). Spikes produced by the neurosecretory cells and muscle potentials of DLMs were differentiated by using spike-sorting software (Spike 2; Cambridge Electronic Design, Cambridge).

RESULTS

Electrical activity of developing flight muscles

EMGs of the developing flight muscles of 10 female Bombyx mori pupae were recorded during the middle and late pupal period. At 4 or 5 days after pupation, the electrical activity of the growing DLMs could be differentiated as small oscillatory potentials of 15–25 Hz superimposed on occasional large potentials of unknown origin (Fig. 1, day 5). A series of oscillatory potentials usually lasted for 5–20 s, and many series of potentials having different temporal structures clustered to form an active phase. Individual active phases lasted for 10–30 min and were separated by 10–30 min of silence (passive phase). As pupal-adult development proceeded, the electrical potentials of muscle activity grew larger, and the duration of the active phases and the interval between successive active phases gradually increased. The increase in the active phase duration was largely due to an increase in the number and duration of individual series of oscillatory potentials (Fig. 1, day 7). The electrical activity of the DLMs began to decline at approximately one day before eclosion (Fig. 1, day 9), and finally ceased before eclosion. The flight-like pattern of the DLMs at the last stage of development was usually regular and quite similar to an activity pattern of the DLMs (30 Hz) that was observed in a flapping adult moth (data not shown).

The developmental profiles of the mean duration of the active phase and the mean period of rhythmic activity of the DLMs in seven different pupae are shown in Fig. 2. An increase in the period and duration was often observed after the onset or termination of illumination. Although there was significant variation among different pupae with respect to the observed activity and termination profiles, the DLM activities in individual pupae achieved their maximal values on day 7 or 8: the mean (±SD) duration of the active phases and the mean (±SD) period of rhythmic activity on day 8 were 38.7±8.7 min and 74.5±7.3 min (n=8), respectively. The generation of electrical activity by the muscles ceased 5–21 h before adult ec dysis.

Activity patterns of different flight muscle bundles

In order to compare the activity patterns of four bundles of indirect flight muscles (right and left DLMs and DVMs), simultaneous EMG recordings were obtained from two synergistic or antagonistic muscle bundles. The records demonstrated that the clusters and sub-clusters of electrical activity in the right and left DLMs (or DVMs) or the ipsilateral pair of DLMs and DVMs were almost perfectly coordinated, although single muscle potentials from different muscle bundles did not always coincide with each other, and a unilateral failure of muscle potentials was often observed (Fig. 3, arrowheads). These results suggest that the rhythmic discharge of electrical activity of all indirect flight muscles may be regulated by a common neural mechanism. Individual muscle potentials from the right and left DLMs were usually synchronized (Fig. 3A, inset), while those from antagonistic muscles (DLM/DVM) were alternate (Fig. 3B, inset). However, such an in-phase or anti-phase relationship was occasionally observed to disappear for a short period of time, even at the last stage of muscle development (data not shown).

Firing activity of neurosecretory cells and its coordination with DLM activity

The rhythmic discharge of flight pattern-like motor activity by the developing flight muscles was closely correlated with the rhythmic firing activity of neurosecretory cells that produce PBAN and are localized in the SG. Because the short- and long-term firing activity profiles of the PBAN-producing cells during the pupal period have been previously

Fig. 2. Developmental changes in flight muscle activity in seven different pupae. The duration of the active phases (A) and the period of rhythmic activity (B) were averaged every 3 h. Different symbols indicate different pupae. Photophase and scotophase are shown by white and black bars at the bottom, respectively.
Fig. 3. Coordinated activity of different muscle bundles on day 9. Series of muscle potentials from the right and left DLMs (A) and from the ipsilateral DLMs and DVMs (B) are illustrated. Each arrowhead indicates a failure of a few muscle potentials in one of the paired muscles. In-phase or anti-phase relationship between the muscle potentials generated by the two synergistic muscles (A) or the antagonistic muscles (B) is shown in the insets. Scale bars: 0.2 mV.

Fig. 4. Typical firing patterns of PBAN-producing neurosecretory cells at three different developmental stages. Scale bars: 0.2 mV.
The simultaneous recordings of DLM activity and neurosecretory cell activity revealed a close relationship between the two rhythmic activities; a cluster of electrical activity in flight muscles always corresponded to a cluster of action potentials of PBAN-producing cells (Fig. 5A). This coordination was observed from days 4–9, after which no muscle potentials were observed. The discharge rates of muscle potentials as well as the firing event rates of neurosecretory cells fluctuated significantly, and there was no apparent correlation between the fluctuations (Fig. 5B). On an expanded time scale, it was evident that the flight muscles became active a few minutes after the neurosecretory cells did (Fig. 6A). On days 6–8, the time delays in the first series of muscle potentials relative to the first action potential of neurosecretory cells were distributed at around 90 s (Fig. 6B). These delays changed daily, and the mean delays (±SD) on days 5, 7, and 9 were 52.9±41.6 s (n=13), 120.6±83.8 s (n=13), and 165.5±97.0 s (n=8), respectively. In contrast, the relative timing of the completion of these activities appeared not to be very strictly regulated, and the muscles often functioned longer (Fig. 6B). There was no apparent daily change in the delayed termination of muscle activity. The developing flight muscles and the neurosecretory cells were closely linked; that is, there were closely parallel daily (developmental) changes in the two activities (Fig. 7). In the most active period, the total duration of activity was approximately 10 h/day.

DISCUSSION
The rhythmic discharge of flight-like motor patterns by developing muscles has been extensively studied in *Manduca sexta*, *Antheraea polyphemus*, and *Antheraea pernyi* (Kammer and Rheuben, 1976; Kammer and Kinnamon,
1979), and the present study revealed that similar electrical activity is generated by the flight muscles of Bombyx mori during postembryonic development. These lepidopteran insects have "synchronous" flight muscles, in that each contraction of these muscles is synchronized with the action potential that initiates it. Many other insects, including flies, wasps, bees, and beetles, have evolved unique, "asynchronous" muscles that can contract with a much higher frequency than the synchronous flight muscles. This is because the individual contractions of an asynchronous muscle are not synchronized with individual nerve action potentials, and the muscle can produce many oscillations in response to each action potential (e.g., Hill et al., 2004). In a preliminary experiment, we recorded similar rhythmic discharges of flight pattern-like motor activity from the developing asynchronous flight muscles of flies (Drosophila melanogaster and Protaphormia terraenovae), the honeybee (Apis mellifera), a wasp (Vespa analis), and a beetle (Tenebrio obscurus), but not from the nymph of a locust (Locusta migratoria) during resting or walking. Thus, the rhythmic electrical activity of flight muscles, irrespective of their muscle types, may be common at least in holometabolous insects, and it plays an important role in the postembryonic development of flight-related neuro-muscular systems. Stevenson and Kutsch (1988) have demonstrated that a larval locust having immobile wing-buds is capable of generating flight-like motor activity in the wing elevator and depressor muscles in response to a wind stimulus. Spontaneous motility is common in many vertebrate embryos, and this embryonic activity appears to be important for the develop-
Neurosecretory Cells and Flight Muscles

Fig. 7. Averaged daily activities of flight muscle (DLMs) and neurosecretory cells (NSCs) during the pupal period. Each bar in the histogram shows the mean and S.E.M (n=5).

ment of neurons and muscles (e.g., Provine, 1984; O'Donovan and Wenner, 1998).

Usually, the flight-like motor activity of the immature flight muscles of *Manduca sexta* does not cause contraction of the muscles (Kammer and Kinnamon, 1979), although repetitive stimuli can evoke tetanus of developing DLMs in the moth *Antheraea pernyi* (Basler, 1969). In a preliminary experiment, we measured the hydrostatic pressure of the thorax of a pharate moth of *Bombyx mori*, but a pharyngomannometer placed in the thorax was unable to detect any change in pressure corresponding to individual muscle potentials. Thus, the developing flight muscles of *Bombyx mori* may be unable to respond mechanically to electrical muscle potentials. An animal having an exoskeleton has to replace the old cuticle with a new one during each molting cycle. If an immature muscle contracts before it forms a tight connection with the new cuticle, it is likely to lead to damage to the muscle itself and/or detachment of the muscle from the cuticle. Therefore, the absence of contractility in immature flight muscles is probably significant for the normal development of flight-related mechanical structures. Detachment and slow progressive retraction of flight muscles to the posterior of the thorax due to muscle hypercontractility have been observed in a mutant of *Drosophila* (Gajewski et al., 2003). In the locust, the neural suppression of muscle tension prevents damage to the legs during the period following molting (Norman, 1995).

The rhythmic activity of the developing flight muscles was closely coordinated with the firing activity of the PBAN-producing cells, and the timing of the onset of the two activities was strictly regulated (Fig. 6). The lead of the neurosecretory cell activity by a few minutes suggests that the prior activation of the neurosecretory cell system may directly or indirectly activate the flight motor system of a pharate moth. If this occurs in vivo, the activation of the motor system may be neural rather than hormonal. This is because bilateral transection of the maxillary nerve (the axonal transport pathway for neurosecretory products) has no effect on the coordinated activity of the two systems, whereas cerebral neural mechanisms appear to be important for the coordination (Kamimoto and Ichikawa, 2006).

Since an elaborate muscular system as well as neuronal control mechanisms of the system are needed for flight, the electrical activity of the developing flight motor system in a pharate moth has been considered to be important for the maturation of muscles, at neuro-muscular junctions, and/or for neuronal pattern-generation mechanisms in the CNS (Kammer and Rheuben, 1976; Kammer and Kinnamon, 1979); however, there is no direct evidence in support of this. In a preliminary experiment, we denervated DLMs uni-laterally during the middle pupal stage (day 5) and compared the muscle contraction kinetics of denervated and innervated (control) muscles after adult emergence. The denervation of the DLMs had a significant effect on the time course of muscle contraction; contraction of the denervated muscles induced by electrical stimulation was significantly slower than that of the control muscles. This suggests that an activity-dependent process may be involved in the maturation of flight muscles during metamorphosis (Kamimoto and Ichikawa, 2004).

PBAN was originally identified as a hormone that acted on the pheromone glands to stimulate sex pheromone production in an adult female moth (Raina and Klun, 1984; Raina et al., 1989). Many related peptides with a common FXPRILamide motif were identified later and classified into the pyrokinin/PBAN family (Nässel, 2002). Peptides belonging to this family have diverse physiological functions, such as modulation of visceral muscle contraction, induction of embryonic diapause in the silkworm, acceleration of puparium formation in flies, stimulation of melanization in some larval moths, and termination of pupal diapause in the moth *Helicoverpa* (Nässel, 2002; Zhang et al., 2004). The close coordination between the firing activity of PBAN-producing cells and the motor activity of developing flight muscles revealed in the present study raises the important question of whether PBAN (and similar neuropeptides) has a hormonal (modulatory) effect on the activity-dependent maturation of flight muscles or on the motor system in pharate moths. Little is known about whether these neuropeptides can act directly on a (developing) skeletal muscle itself or the neuromuscular junctions of the muscle. Leucopyrokinin, a peptide belonging to the pyrokinin/PBAN family, can induce longitudinal body contractions that are required for puparium formation in fleshfly larvae, but the target for the neuropeptide is the CNS and not the peripheral nervous system (Zdárek et al., 2002). G-protein-coupled receptors for PBAN have been identified in the moths *Helicoverpa zea* (Choi et al., 2003) and *Bombyx mori* (Hull et al., 2004), and the distribution of PBAN receptors may facilitate the elucidation of the functional roles of the peptide in the development of imaginal structures, including flight muscles.

The median neurosecretory cells in *Manduca sexta* are
immunoreactive to antisera against other neuropeptides, including proctolin and FMRFamide (Davis et al., 1996); these neuropeptides are effective on skeletal muscles in insects and crustaceans (Gàde et al., 1997; Konopinska and Rosinski, 1999; Orchard et al., 2001). Proctolin and FMRFamide-related peptides (FaRPs) are usually released from the nerve endings of motor neurons as cotransmitters of glutamate, to modulate muscle contractions via pre- and/or postsynaptic mechanisms (Gàde et al., 1997; Konopinska and Rosinski, 1999; Orchard et al., 2001). These neuropeptides are present in many central and peripheral neurosecretory cells and neurohemal sites, thereby suggesting their role as circulating hormones (Nàssel, 2002). Neurally evoked contractions in the DLMs of the moth Manduca sexta are potentiated by the application of the pentapeptide FaRP (FLRFamide) (Kingan et al., 1990). Injection of proctolin into a pupa of the mealworm beetle Tenebrio molitor rapidly potentiated the amplitude of hemocoelic pulsations caused by rhythmic contractions of the abdominal intersegmental muscles (Slàma et al., 1993). We revealed that rhythmic firing activity by the median neurosecretory cells of the SG in the mealworm Tenebrio obscurus pupa was closely coordinated with periodically occurring rhythmic abdominal contractions; this suggests that the neuropeptides released from the neurosecretory cells probably act on the abdominal intersegmental muscles to increase the amplitude of contractions (Ichikawa and Inoue, 2006). Because rhythmic abdominal contractions or pulsations play an important role in hemolymph circulation and ventilation during the pupal period (Slàma, 2000), the myotropic neuropeptides of the beetle may indirectly promote the growth of imaginal tissues by stimulating physiological systems related to energy supply and the transport of metabolic substrates and wastes. The physiological actions of these myoactive peptides on the (developing) skeletal muscles in the silkmoth and beetle remain to be examined.

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