Cellular events and molecular mechanisms underlying sex pheromone production in the silkmoth, *Bombyx mori*

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Lepidopteran sex pheromones are aliphatic compounds with limited functional groups, and are synthesized *de novo* in the pheromone gland from acetyl-CoA through the fatty acid biosynthetic pathway. A neurohormone termed pheromone-biosynthesis-activating neuropeptide (PBAN) regulates sex pheromone production in a number of lepidopteran species. With the aim of promoting general understanding, we review recent studies on the cellular events and molecular mechanisms of sex pheromone production in the silkmoth, *Bombyx mori*.

Introduction

Insects have developed diverse systems during the process of evolution, and in these systems specific lipid compounds are involved in the regulation of various physiological events. For example, ecdysteroids (steroids) and juvenile hormones (terpenoids) have significant roles in the development, metamorphosis, and reproduction of insects. Various pheromones with different functions also play a significant role as a medium of communication between individuals. These lipid compounds are synthesized and released from specific gland cells. The main aim of our research is to elucidate molecular mechanisms underlying the biosynthesis, transportation, and release of such lipids from the cells producing them using insect systems. In this paper, we will review current understanding of the sex pheromone production in lepidopteran insects from cellular and molecular aspects.

Sex pheromones in Lepidoptera

Many species of moths have their own pheromone blends that serve as a medium of communication in mating behavior, and structural/compositional variations of the pheromone blends are responsible for species specificity. Sex pheromones produced by female moths are generally C_{12}-C_{18} unsaturated acyclic aliphatic compounds with limited functional groups such as aldehyde, alcohol, and acetate ester. These sex pheromone components are synthesized *de novo* in the pheromone gland from acetyl-CoA: through the fatty acid biosynthetic pathway, palmitate (16: Acyl) or stearate (18: Acyl) is converted into saturated fatty acyl derivatives of different chain length such as 18: Acyl, 16: Acyl, 14: Acyl, and 12: Acyl, which undergo a desaturation step catalyzed by a Δ^{11} desaturase, or vice versa. Different combinations of chain shortening and desaturation steps catalyzed by a Δ^{11} desaturase or other desaturases produce various proportions of the known lepidopteran sex pheromone components.[1)]

In *Bombyx mori*, the sex pheromone bombykol, (E,Z)-10,12-hexadecadien-1-ol, is synthesized through palmitate (16: Acyl), which is stepwise converted into bombykol by Δ^{11} desaturation, $\Delta^{10,12}$ desaturation, and fatty acyl reduction.[2)]

Regulation of pheromone biosynthesis

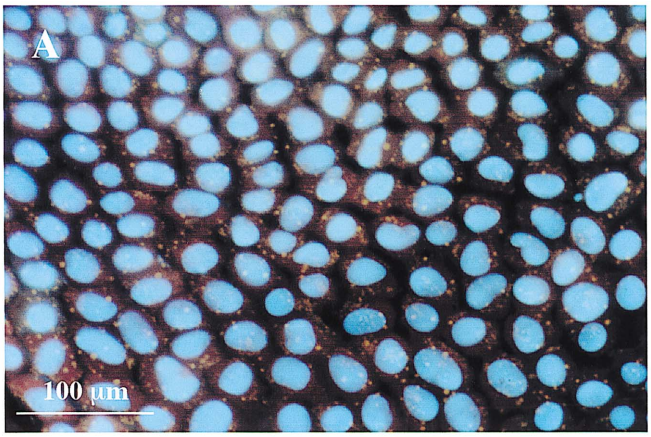
In a number of lepidopteran species, a neurohormone termed pheromone-biosynthesis-activating neuropeptide (PBAN) originating from the suboesophageal ganglion stimulates sex pheromone production. The biochemical step regulated by PBAN in the pheromone biosynthetic pathway is either fatty acyl reduction or that prior to fatty acid synthesis depending on the moth species.

In *B. mori*, PBAN directly acts on the pheromone gland and regulates the fatty acyl reduction step, which is the final step of the bombykol biosynthetic pathway. One of the key enzymes under PBAN control is acyl-CoA reductase.[3)]

Pheromone-producing cells

In female Lepidoptera, the pheromone gland, a functionally specialized organ producing a species-specific sex-pheromone blend, is a modified intersegmental membrane generally located between the eighth and ninth abdominal segments. The epidermal cells of the pheromone gland are in direct contact with the overlying cuticle, and it is believed that the pheromone components produced within the cells are transported through the cuticle and disseminated from the surface of the cuticle.[4)]

In *B. mori*, the pheromone gland is represented as a pair of eversible, ventrolateral sacs (*sacculi laterales*), and begins to produce bombykol after adult eclosion in response to PBAN. Bombykol-producing cells are homogeneous epidermal cells consisting of about 10000 cells/gland. The most distinct characteristic feature of bombykol-producing cells is the accumulation of many refractile granules within their cytoplasm (Fig. 1). Since the granules are stained with Nile Red, these refractile granules are considered to be lipid droplets.[5)]



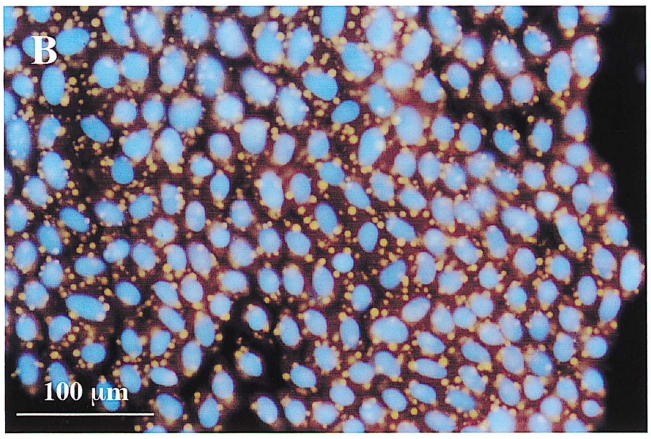


Fig. 1. Pheromone-producing cells of *B. mori* 2 days (A) and 1 day (B) before adult eclosion. Lipid droplets (yellow) were stained with Nile Red while nuclei (blue) were stained with Hoechst 33342.

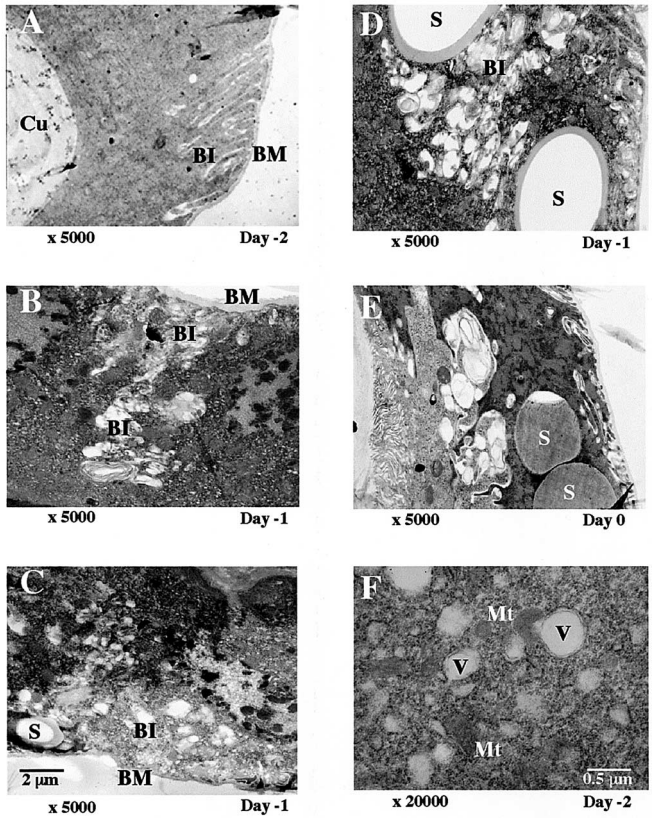


Fig. 2. Electron microphotographs of bombykol-producing cells 2 days (A, F), and 1 day (B-D) before eclosion, and on the day of eclosion (E). BI, basal involution; BM, basement membrane; Cu, cuticle; S, sphere; Mt, mitochondrion; V, small vesicle.

Cellular events in pheromonogenesis

Accumulation of lipid droplets within the cytoplasm is a common feature of the pheromone gland cells of lepidopteran species. However, the role of the lipid droplets in pheromone production has not been clarified. We have investigated morphometrical changes of the lipid droplets in relation to bombykol production induced by pheromonotropic stimuli. Cytoplasmic lipid droplets fluctuated in both size and number after adult eclosion. These morphometrical changes, however, were prevented by decapitation and reversed by application of a pheromonotropic stimulus, i.e., by PBAN injection.[5, 6] Preliminary extraction and chemical analysis of the lipid droplets indicated that they are a mixture of triacylglycerols (TGs) with the putative bombykol precursor, $\Delta^{10,12}$ hexadecadienoate, as a major component. These properties of the lipid droplets and cellular dynamics associated with the external signal of PBAN suggest a storage-pool function of the lipid droplets in which bombykol precursors accumulate in the form of TG.

Using light and electron microscopies, we have demonstrated that the lipid droplets appear starting 2 days before adult eclosion, and a marked accumulation occurs between 2 days and 1 day before eclosion (Fig. 1). On the day of eclosion, mature lipid droplets were observed by electron microscopy as full electron-dense spheres 4 to 10 μm in diameter. Since we observed peripherally or partially electron-dense spheres of similar size to the mature lipid spheres 1 day before eclosion, we assume that they are immature forms of the lipid droplets (Fig. 2C-E). In addition, since we observed that production of these spheres occurred in association with extensive involutions in the basal plasma membrane (Fig. 2A-D), this finding suggests that the basal involutions are closely related to the formation of cytoplasmic lipid droplets. More importantly, the basal involutions also suggest an extracellular origin of the lipid droplet contents. In our analysis of the composition of TGs in the lipid droplets, we have detected oleic, linoleic, and linolenic acids in addition to bombykol biosynthetic intermediates, hexadecenoic and hexadacadienoic acids. In *B. mori*, it has been nutritionally demonstrated that linoleic and linolenic acids are essential fatty acids, and accordingly they have to be taken up in the diet by this species. Considering these findings in *B. mori*, the basal involutions into bombykol-producing cells seem to reflect the uptake of the extracellular lipid components including linoleate or linolenate, which are required for the construction of TGs containing *de novo*-synthesized bombykol precursors.

In *B. mori*, bombykol is synthesized from acetyl-CoA via the palmitic acid intermediate, which is generally synthesized by the action of cytosolic fatty acid synthase, and is converted to palmitoyl-CoA by acyl-CoA synthetase in the outer membrane of mitochondria. By electron microscopy, we observed

numerous small vesicles less than 1 μm in diameter in the cytoplasm, some of which were fused to mitochondria preceding the lipid droplet formation 2 days before adult eclosion. Thus the fused structures of the small vesicles and mitochondria seem to be a manifestation of the above-described biochemical process (Fig. 2F).

Molecules involved in bombykol production

Since PBAN directly acts on the pheromone gland, the entire biochemical process regulated by PBAN has to occur in the pheromone gland. In order to probe the biochemical steps as well as the underlying mechanisms regulated by PBAN, we have tested the effects of various enzyme inhibitors/activators on the production of bombykol using a gland incubation assay.[1)] Our results suggest that the PBAN signal transduction cascade involves the influx of extracellular Ca^{2+}, formation of a Ca^{2+}/calmodulin complex, activation of calcineurin, and activation of acyl-CoA reductase by dephosphorylation. To elucidate the precise mechanisms underlying sex pheromone production in *B. mori*, we have cloned the genes of a pheromone-gland-specific acyl-CoA desaturase, two acyl-CoA-binding proteins predominantly expressed in the pheromone gland,[7, 8)] and heterosubunits of calcineurin from a pheromone gland cDNA library. We are now attempting unequivocal demonstration of the role of these molecules during pheromonogenesis.

We have also constructed a cell-free system for producing bombykol using a microsomal fraction of the gland cells and indicated that the reduction of the acyl group, which is the final step of bombykol biosynthesis, is catalyzed by two sequential enzymes, acyl-CoA synthetase and acyl-CoA reductase.[9)] Recently, we have cloned a gene encoding a protein similar to the plant seed reductase, jojoba fatty acid reductase. Northern blot analyses revealed that the transcript was predominantly expressed in the pheromone gland and the expression occurred markedly 1 day before adult eclosion in association with that of the gland-specific acyl-CoA desaturase. These findings suggest that the clone encodes the acyl-CoA reductase involved in the final step of bombykol biosynthesis.

Prospects

As part of the genome project in *B. mori*, the construction of *B. mori* EST (expressed-sequence-tag) database started in 1996.[10)] At present, more than 7500 independent genes of *B. mori* have been tagged from cDNA libraries prepared from various tissues including the pheromone gland. Using the EST database, we can configure the genes abundantly expressed in the tissue from which the cDNA library is constructed, and thus the application of this methodology would be useful in elucidating the molecular background of some specific events such as pheromone production. However, there remains a limitation to clarify the precise function of each gene, even if we can identify various genes involved in the specific events by this methodology. This is mainly because efficient and practical transgenic systems for studying gene functions have not been established in most insects other than in *Drosophila*.

In order to overcome this limitation, we are now constructing an efficient system for delivering a gene directly to the target cells of interest by infecting *B. mori* with a recombinant baculovirus. At present, viral vectors are the most efficient tools for gene delivery in mammals. Since baculovirus is widely used as a vector for expression of foreign genes in insect cells, recombinant baculoviruses could serve as gene-transfer vehicles for the transient expression of recombinant proteins in a wide range of insect tissues. In addition, using tissue-specific promoters and GFP as a fluorescent marker, this system would contribute to the elucidation of gene functions as well as the cellular dynamics of biological events in insects. Thus the gene-delivery system assisted by baculoviruses could become a powerful tool for the study of molecular mechanisms in insects, similar to the adenovirus-gene-delivery system used in mammals. Thus, in this program, we employ a molecular biological approach associated with such novel methodologies for full understanding of the molecular mechanisms underlying diverse biological events in insects.

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