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ULTRASTRUCTURAL STUDIES ON THE PHEROMONE-PRODUCING CELLS IN THE SILKMOTH, *BOMBYX MORI:* FORMATION OF CYTOPLASMIC LIPID DROPLETS BEFORE ADULT ECLOSION

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In *Bombyx mori,* pheromone-producing cells accumulate a number of lipid droplets in the cytoplasm preceding the production of the sex pheromone, bombykol. The process of lipid droplet formation in the pheromone-producing cells was investigated by using light and electron microscopy. Light microscopy revealed that the lipid droplets appeared from 2 days before adult eclosion and dramatic accumulation took place between 2 days and 1 day before eclosion. Electron microscopical studies revealed that smooth endoplasmic reticulum and numerous vesicles, their sizes being less than 1 μm, were detectable 2 days before eclosion, and some vesicles were fused with mitochondria at this stage. These characteristic changes in the pheromone-producing cells suggest that fatty acyl-CoA synthesis following *de novo* fatty acid synthesis takes place at this time. Involutions in the basal plasma membrane of the cells occurred throughout the observed period, which were extensive on the day before adult eclosion. Besides extensive basal involutions, immature lipid droplets appeared and then mature fully electron-dense lipid droplets were observed on the day of adult eclosion. These ultrastructural observations, combined with recent physiological studies suggest, that the basal involutions presumably reflect the uptake of lipidic components required for the construction of lipid droplets, the function of which is to store the bombykol precursor and to provide it for bombykol biosynthesis in response to pheromonotropic stimuli by pheromone biosynthesis activating neuropeptide (PBAN).

Keywords: Bombyx mori – pheromone biosynthesis – lipid droplets – basal involution – PBAN

INTRODUCTION

In female Lepidoptera, the pheromone gland (PG), a functionally specialized organ to produce a species-specific sex-pheromone blend, is a modified intersegmental membrane-like structure generally located between the eighth and ninth abdominal segments. The epidermal cells of the PG are in direct contact with the overlying sur-

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face cuticle (lamellate endocuticle, protein epicuticle, a thin electron-dense cuticulin layer, and outer epicuticle), and it is believed that the pheromone components are produced within the cells, transported through the cuticle, and disseminated from the surface of the cuticle [17, 20]. In lepidopteran PGs, epidermal cells are hypertrophied and the histological features characteristic of these cells are a large nucleus, many vacuoles, and sometimes an apical "brush border" [18]. In addition, ultrastructural studies have revealed that they contain smooth endoplasmic reticulum (ER) – often tubular –, lipid droplets, microvilli, and apical folds (see for *Bombyx mori* [25, 27], for *Plodia interpunctella* [24], for *Choristoneura fumiferana* [14] and review [18]). Quite recently, a complete ultrastructural study of the PG in relation to pheromone production and release was conducted in *Helicoverpa zea* [20].

Female-produced lepidopteran sex pheromones are generally biosynthesized from fatty acids through desaturation and chain-shortening reactions followed by reductive modification of the carbonyl carbon (see reviews [22, 26]). The biosynthesis is often regulated by a neurohormone termed pheromone biosynthesis activating neuropeptide (PBAN) originating from the suboesophageal ganglion, and the intracellular signal transduction cascade caused by PBAN has been investigated in various moth species [9, 12, 19]. Despite the fact that much physiological as well as biochemical research has been directed towards a better understanding of pheromone production in lepidopteran insects, far less is known about the relationships between cellular dynamics and pheromone production.

In the silkmoth, *B. mori*, the sex pheromone bombykol {(E, Z)-10,12-hexadecadiene-1-ol} is produced in the PG represented as a pair of eversible, ventrolateral sacs (*sacculi laterales*) [18]. The epidermal cells of the gland are cuboidal, contain smooth tubular ER, and have apical folds [25, 27]. The most obvious characteristic feature of these pheromone-producing cells is, however, the accumulation of lipid droplets (or vacuoles) within the cytoplasm. Hayashi and Ito [7] first reported the presence of vacuoles in the PG of this species. Steinbrecht [25] carried out detailed histochemical tests on the vacuoles, and concluded that they were not likely to be the sex attractant bombykol, although they contained unsaturated neutral lipids. Later, Waku and Sumimoto [27] conducted ultrastructural studies on the PG and reported that the lipoid substances appear in the cells 4 days before eclosion and they originate from characteristic myelin figures, the mitochondria being the starting orgenelle of such structures. Based on electron microscopical studies, they assumed that the lipoid substances represent the secretory substance of the cell also containing bombykol.

After a long intermission, we recently investigated morphometrical changes of the lipid droplets in relation to bombykol production caused by pheromonotropic stimuli [5, 6]: Cytoplasmic lipid droplets fluctuated both in size and in number after adult eclosion. These morphometrical changes, however, could be prevented by decapitation and reversed by pheromonotropic stimulus, i.e. by PBAN injection. The finding that the lipid droplets absorb Nile Red indicates that they contain neutral lipids [5]. These observations on the lipid droplets and cellular dynamics associated with the external signal of PBAN in the PG suggested a storage-pool function of the lipid droplets where bombykol precursors accumulate. In the present paper, we describe

electron microscopical studies on the pheromone-producing cells of the silkmoth, *B. mori*, especially focussing on the lipid droplet formation before adult eclosion, and discuss the cellular events in relation to bombykol biosynthesis preceding the pheromonotropic stimuli by PBAN.

MATERIALS AND METHODS

Insects and tissue dissection

Eggs of *B. mori* (Shuko X Ryuhaku) were purchased from Katakura Kogyo and larvae were reared on an artificial diet as described previously [4]. Pupal age was determined based on the following morphological characters: Eye pigmentation occurs three days before adult eclosion. Further pigmentation takes place in the antennae 2 days before eclosion and comb-like structure appears in the antennal area 1 day before eclosion. After removal of pupal cuticle, each abdominal tip was excised and the PG corresponding to the 8–9th intersegmental membrane was trimmed in the Ringer solution [35 mM NaCl, 36 mM KCl, 12 mM $CaCl_2$, 16 mM $MgCl_2$, 274 mM glucose, and 5 mM Tris-HCl (pH 7.5)] as described previously [11]. Trimmed PGs were subjected to various microscopical studies.

Light microscopy

Following rinsing in phosphate-buffered saline (PBS), trimmed PGs were fixed for 10 min in PBS containing 2.5-fold diluted 10% formalin neutral buffer solution (Wako Pure Chemicals, Tokyo). The fixative was removed and the glands were rinsed three times with PBS. After rinsing the specimen was covered, sealed, and subjected to light microphotography. Bright field images were obtained with OLYMPUS BX-60 system equipped with PM-30 exposure unit (1000× magnification). Samples were prepared from the pupae 3, 2 and 1 day(s) before eclosion or from the newly emerged females on the day of eclosion.

Electron microscopy

Sample preparation for electron microscopy was performed as described before [27]. Trimmed PGs rinsed in PBS were fixed at 4 °C for 12 h in 50 mM cacodilate buffer (pH 7.4) containing 0.17 M sucrose and 5% glutaraldehyde. After rinsing overnight in the buffer, the glands were post-fixed with 1 % osmium tetroxide for 1 h and dehydrated in a series of aqueous ethanol. The glands were then embedded in Quetol 812 (Nissin EM Co. Ltd.). Ultra-thin sections (100 nm thickness) were prepared with a glass knife on a LKB ultramicrotome. After placing specimens on copper grids, they were doubly stained for 5 min with saturated uranyl acetate for 30 min and then with

Reynold's [21] lead citrate for 6 min. Observations and microphotograpy were performed with JEOL 1200EX or JEOL 1010 electron microscope. Trimmed PGs were dissected from pupae 2 days and 1 day before eclosion or from female moths on the day of eclosion. Dissection was performed daily in the photophase (1 h after light on).

RESULTS

Appearance of cytoplasmic lipid droplets in the pheromone-producing cells

In order to monitor the appearance of cytoplasmic lipid droplets, first, the pheromone-producing cells prepared from *B. mori* pupae were examined at different developmental stages before adult eclosion by using light microscopy. Figures 1A–D show the process of lipid-droplet formation from 3 days before adult eclosion until the day of eclosion. Three days before adult eclosion, boundaries of pheromone-producing cells were observed under the processes on the cuticular surface, but no lipid droplets were present in the cytoplasm (Fig. 1A). In contrast, some lipid droplets appeared at 2 days before eclosion, their diameters being less than 5 μm (Fig. 1B). The cell boundaries of the pheromone-producing cells were still detectable at this stage. A dramatic accumulation of large lipid droplets took place on the day before

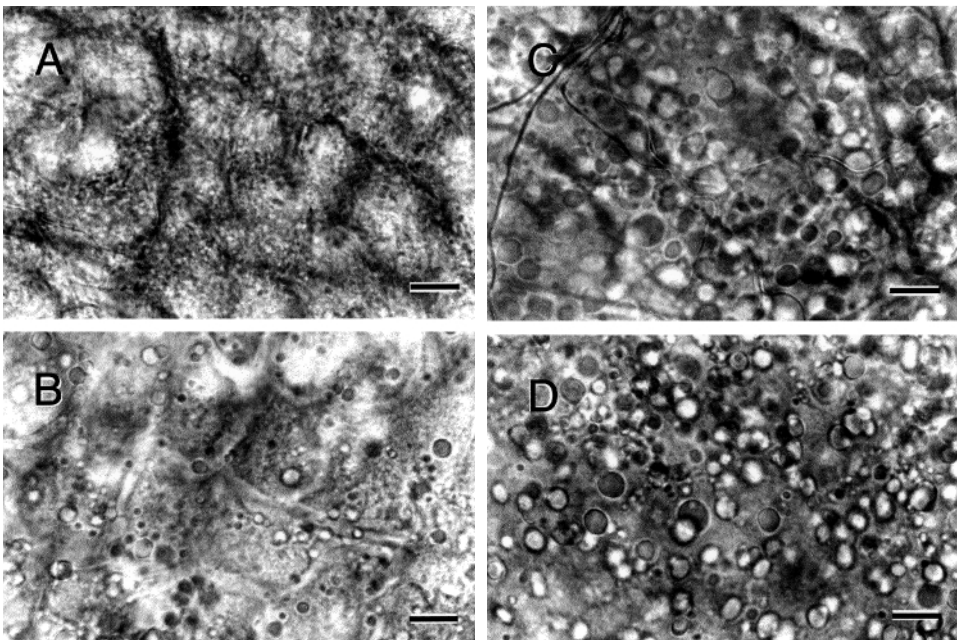

Fig. 1. Light microphotographs of the pheromone gland cells taken from *B. mori* pupae. A: 3 days before eclosion; B: 2 days before eclosion; C: 1 day before eclosion; D: soon after eclosion. Bars: 10 μm

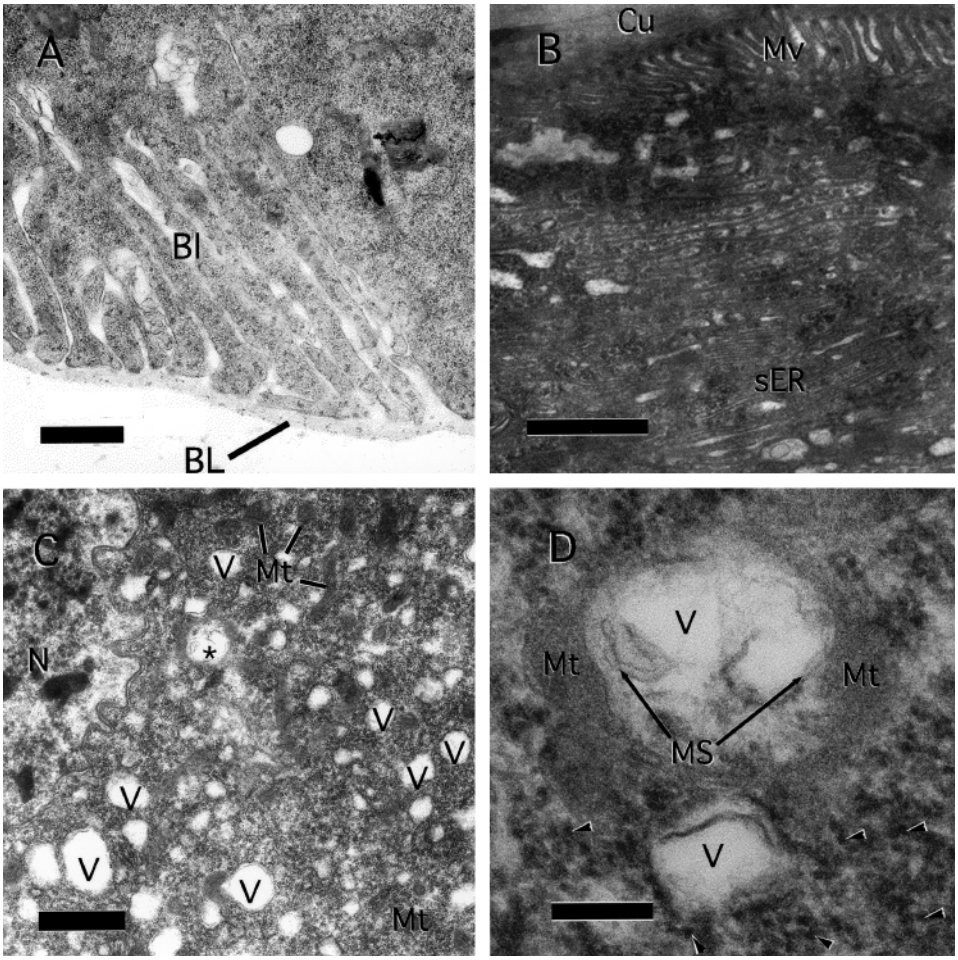

Fig. 2. Electron microphotographs of *B. mori* pheromone gland cells 2 days before eclosion. A: Basal region of the gland cell. Bar: 1 μm; B: Apical region of the gland cell. Microvilli are present on the entire surface of the cuticular side. Bar: 1 μm; C: Cytoplasmic region of the gland cell. Numerous vesicles are present in the vicinity of mitochondria, some of which are fused to the vesicles *(asterisk)*. Bar: 1 μm; D: High magnification of the cytoplasmic region of the gland cell. Arrowheads indicate ribosomes. Bar: 0.25 μm (BI: basal involution; BL: basal lamina; Cu: cuticle; Mt: mitochondria; Mv: microvilli; N: nucleus; sER: smooth endoplasmic reticulum; MS: membrane structure;V: vesicle)

eclosion (Fig. 1C) and the accumulation continued until the day of adult eclosion (Fig. 1D). The lipid droplets in this stage typically varied from 4 to 10 μm in diameter, and those larger than 5 μm were characteristic. The cell boundaries were not recognized probably due to the abundance of cytoplasmic lipid droplets. Since the lipid droplets appear from 2 days before adult eclosion, we examined the details of the droplet formation by means of electron microscopy thereafter.

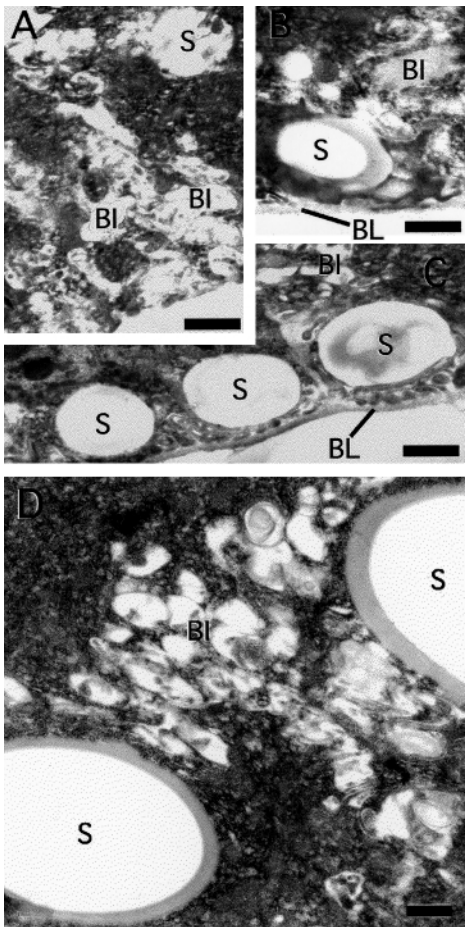

Fig. 3. Electron microphotographs of *B. mori* pheromone gland cells 1 day before eclosion. A: Characteristic spheres are present in the vicinity of irregular-shaped involutions from the basal plasma membrane; B: A spherical part with clear boundary is formed among similarly prominent involutions; C: Spherical structures with sizes similar to the spherical parts are formed. One of them contains some material with electron density; D: Large spheres among involutions with peripherally electron-dense material. Bars: 1 μm (BI: basal involution; BL: basal lamina; S: sphere)

Electron microscopy observations during the process of lipid droplet formation

Organelles and cellular dynamics during the process of lipid droplet formation was examined in the pheromone-producing cells prepared from individuals 2 days and 1 day before adult eclosion as well as on the day of eclosion (Figs 2–4, respectively). In specimens 2 days before eclosion, basal plasma membrane of the cells opposing

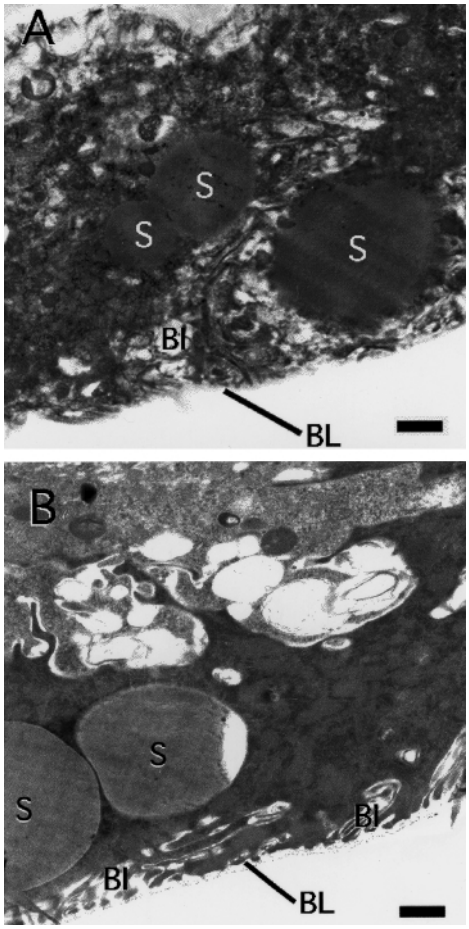

Fig. 4. Electron microphotographs of *B. mori* pheromone gland cells at adult eclosion. A: Fully electron-dense spheres; B: Almost, but not fully electron-dense spheres with similar size also exist surrounded by electron-dense cytosol. Large intercellular spaces lie between the cells. Bars: 1 μm (BI: basal involution; BL: basal lamina; S: sphere)

the overlying basal lamina had basal involusions (Fig. 2A), and free ribosomes numerously occupied the cytoplasm between other structures and organelles (Fig. 2D). These two features were present throughout the examined period. Smooth ER with horizontally aligned long cisternae at the apical part of the pheromone-producing cells was also observed at this stage (Fig. 2B), but neither a day before eclosion, nor at adult eclosion. Rod-shaped mitochondria with a dense matrix and flat lamellar cristae were abundant, and numerous electron-thin vesicles with size less than 1 μm were observed in the cytoplasm (Fig. 2C). In addition, structures of some vesicles fused with mitochondria were seen at this stage (Figs 2C, D).

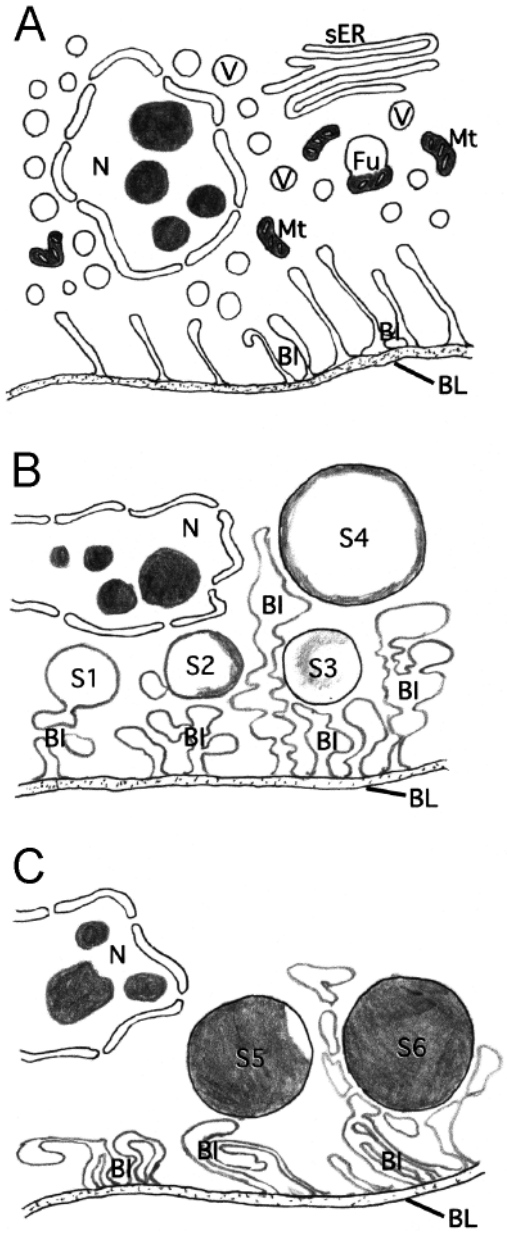

Fig. 5. Schematic representation of suggested intracellular dynamics in the pheromone gland; the development and maturation of lipid droplets. A: Two days before eclosion; B: One day before eclosion; C: Soon after eclosion. (BI: basal involution; BL: basal lamina; Fu: fusion of low electron-density vesicles and mitochondria; Mt: mitochondria; N: nucleus; S1–S6: sphere, where the numbering is indicating consecutively the developmental phase; V: vesicle)

On the day before eclosion, the involutions in the basal plasma membrane observable in Fig. 2A got enlarged and prominently extended into the cytoplasm of the cell (Fig. 3A). These irregular-shaped, but growing structures on the basal involutions suggest an active role, dynamism. Furthermore, among these basal involutions, spheres of various sizes appeared (Figs 3A–D). Although these spheres were basically with low electron density similar to those of basal involutions, some of the firm ones contained areas of irregular electron-density (Figs 3B, C). Figure 3D shows such a typical example of large spheres with ring-like distribution of electron density.

On the day of adult eclosion, many fully electron-dense spheres could be observed (Fig. 4A), the size of which corresponded to the cytoplasmic lipid droplets seen in Fig. 1D. At this stage almost, but not fully electron-dense spheres with similar size may be detected as well (Fig. 4B). These findings suggest that the fully electron-dense spheres are the mature lipid droplets, but the spheres partially with low electron density observed in Figs 2–3 correspond to the transient states of immature lipid droplets.

The schematic representation of Fig. 5 summarizes the cellular dynamics of the pheromone-producing cells during the process of lipid-droplet formation, which may be suggested based on the presented electron microscopical observations described above. The development and maturation of the lipid droplets (spheres) are indicated with consecutive numbering (S1–S6) on Figs 5B and C.

DISCUSSION

We have investigated the process of lipid droplet formation in the pheromone-producing cells of the female *B. mori,* by using light and electron microscopy. Light microscopy revealed dramatic proliferation of the lipid droplets between 2 days before eclosion and 1 day before eclosion. Electron microscopy studies revealed smooth ER, fusion of mitochondria and vesicles, basal involutions, partially or fully electron-dense granules, and intermediate figures between involutions and the granules. We will discuss below these ultrastructural findings in relation to the underlying biochemical processes of sex pheromone biosynthesis in *B. mori*.

Formation, maturation, and function of cytoplasmic lipid droplets

We have previously reported that bombykol-producing cells in the PG of *B. mori* are homogeneous epidermal cells with many refractile granules in the cytoplasm [5, 6]. Since the granules are stained with Nile Red, these refractile granules proved to be lipid droplets [5]. Accumulation of the lipid droplets (or spheres) within the cytoplasm is a common feature of the PG cells in Lepidoptera, and the accumulation after adult eclosion has been described in various species [3, 16]. However, maturation process of the lipid droplets before (and after) adult eclosion has not been well char-

acterized, and the role of the lipid droplets in the pheromone production has not been clearly understood.

In the present paper we have demonstrated that the lipid droplets appear from 2 days before eclosion, and dramatic accumulation takes place between 2 days before eclosion and 1 day before eclosion (Fig. 1). On the day of eclosion, mature lipid droplets were observed as fully electron-dense spheres with size from 4 to 10 μm in diameter (Figs 1 and 4A). Since we observed peripherally or partially electron-dense spheres with a similar size to the mature lipid spheres on the day before eclosion (Fig. 3), we assume that they are immature forms of the lipid droplets. In addition, since we observed that production of these spheres took place in association with extensive involutions in the basal plasma membrane (Fig. 3), this finding suggests that the basal involutions may be closely related to the formation of cytoplasmic lipid droplets. Furthermore, the extensive involutions in the basal plasma membrane also suggest extracellular origin of the lipid droplet contents.

As for the role of lipid droplets, we have reported that presence or absence of pheromonotropic stimuli greatly influenced the size and number of cytoplasmic lipid droplets after adult eclosion, in *B. mori* [5]. We have also observed reverse correlation between accumulation of large lipid droplets and bombykol production [6]. Until the present days, the chemical nature of cytoplasmic lipid droplets in lepidopteran PGs has not been characterized and only detailed histochemical tests on the droplets in *B. mori* concluded that they contain unsaturated neutral lipids, but not the sex attractant bombykol [25]. Our very recent studies, however, based on the 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 [13]. These results suggest a storage-pool function of the lipid droplets where bombykol precursors accumulate in the form of TGs.

It was reported in *C. fumiferana* [14] and in *Trichoplusia ni* [16] that the basal plasma membrane of PG cells have extensive basal involutions, the function of which is presumably to provide a larger surface area for the uptake of pheromone precursors. Recently, extensive invaginations that are likely to arise from the basement membrane in PG cells of *H. zea* during photophase were also reported, while, on the other hand, no changes in the number and size of lipid droplets between photo- and scotophase were observed in this species [20]. In our analysis regarding the composition of the TGs in the lipid droplets, however, we have detected oleic, linoleic, and linolenic acids, in addition to the bombykol biosynthetic intermediates, hexadecenoic and hexadacadienoic acids [13]. 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 [8]. When we consider 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 the cabbage looper, *T. ni,* the size and distribution of lipid spheres correlated very well to the age of the female and the pheromone content of the gland, and the

spheres moved into the cuticle [16]. These observations led to the assumption that some of the final secretion material, i.e., the pheromone, is in the lipid spheres [16]. In that paper, however, the precise relationship between the changes of lipid spheres and pheromone content with or without pheromonotropic stimuli could not be elucidated, because hormonal regulation of sex-pheromone production in Lepidoptera had not yet been discovered at that time. In addition, since there is no evidence that the lipid spheres of *T. ni* actually contain the pheromone components, it is unclear that the lipid spheres are the storage structures of final secretion in this species.

In the ultrastructural studies on the PG cells of *B. mori*, characteristic myelin figures in the cytoplasm 7 days after pupation (i.e. 4 days before adult eclosion) were reported [27]. Since it was observed that myelin figures contained some low electron-density material, it was assumed that the myelin figures are the precursor material of the lipid droplets, and concluded that mitochondria differentiate into lipid droplets through myelinated mitochondria and myelin figures. However, we could confirm neither myelinated mitochondria nor myelin figures during the period of lipid droplet formation from 2 days before eclosion to the day of eclosion.

Characteristic organelles in the bombykol-producing cells

The specific involvement of the organelles in the biosynthesis and secretion of the sex pheromones has not been determined for most pheromone-producing cells. Development of smooth ER is a typical feature of lipid-producing cells both in mammals and epidermal gland cells of insects and microbodies are also present in such type of cells [14, 18]. In the PG cells of Lepidoptera, smooth ER is tubular or cisternal, and may be located either between the nucleus and the apical plasma membrane or throughout the entire cell [10, 14, 23, 25]. In the white tussock moth *Orgyia leucostigma*, both cisternal and tubular smooth ER are distributed throughout the cell [15]. In *B. mori*, it was reported that the gland cells contain abundant smooth ER, which appear at the time of emergence [25]. In the present study, we have observed multi-lamellar cisternal smooth ER located at the apical portion of the PG cell 2 days before adult eclosion (Fig. 2A). Recently, we have cloned a cDNA encoding PG-specific acyl-CoA desaturase. Acyl-CoA desaturases with two double-membrane spanning regions are located on the smooth ER membrane and the mRNA of the PG-specific acyl-CoA desaturase is prominently expressed from the day before adult eclosion [28]. In addition, the cytoplasmic lipid droplets are produced from 2 days before eclosion and dramatic accumulation takes place thereafter (Fig. 1). Thus, these cellular events in bombykol-producing cells are likely to be accompanied by the appearance of smooth ER in the PG cells.

In *B. mori*, bombykol is *de novo* synthesized from acetyl-CoA via fatty acid biosynthesis [2]. Palmitic acid, the intermediate of bombykol biosynthesis, is generally synthesized by the action of fatty acid synthase in the cytosol, and is converted to palmitoyl-CoA by acyl-CoA synthetase in the outer membrane of mitochondria [1]. We observed numerous small vesicles less than 1 μm in diameter in the cyto-

plasm (Fig. 2C), some of which were fused to mitochondria preceding the lipid droplet formation 2 days before adult eclosion (Fig. 2D). The fused structures of the small vesicles and mitochondria therefore seem to be the manifestations of the above described biochemical process.

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